Developmental differences in locomotor responsiveness to amphetamine in rats

by

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General Abstract

The developmental remodelling of motivational systems that underlie drug dependence and addiction may account for the greater frequency and severity of drug abuse in adolescence compared to adulthood. Recent advances in animal models have begun to identify the morphological and the molecular factors that are being remodelled, but little is known about the culmination of these factors in altered sensitivity to psychostimulant drugs, like amphetamine, in adolescence. Amphetamine induces potent locomotor activating effects in rodents through increased dopamine release in the mesocorticolimbic dopamine system, which makes locomotor activity a useful behavioural marker of age differences in amphetamine sensitivity. The aim of the thesis was to investigate the neural basis for age differences in amphetamine sensitivity with a focus on the nucleus accumbens and the medial prefrontal cortex, which initiate and regulate amphetamine-induced locomotor activity, respectively.

In study 1, I found pre- and post- pubertal adolescent rats to be less active (i.e., hypoactive) than adults to a first injection of 0.5, but not of 1.5, mg/kg of intraperitonealy (i.p.) administered amphetamine. Although initially hypoactive, only adolescent rats exhibited an increase in activity to a second injection of amphetamine given 24 h later, indicating that adolescents may be more sensitive to the rapid changes in amphetamine-induced plasticity than adults. Given that the locomotor activating effects of amphetamine are initiated in the nucleus accumbens, age differences in response to direct injections of amphetamine into this brain region were investigated in study 2. In contrast to i.p. injections, adolescents were more active than adults when amphetamine was given

directly into the nucleus accumbens, indicating that hypoactivity may be attributed to the development of regulatory regions outside of the accumbens.

The medial prefrontal cortex (mPFC) is a key regulator of the locomotor activating effects of amphetamine that undergoes extensive remodelling in adolescence. In study 3, I found that an i.p. injection of 1.5, and not of 0.5, mg/kg of amphetamine resulted in a high expression of c-fos, a marker of neural activation, in the prelimbic mPFC only in pre-pubertal adolescent rats. This finding suggests that the ability of adolescent rats to overcome hypoactivity at the 1.5 mg/kg dose may involve greater activation of the prelimbic mPFC compared to adulthood. In support of this hypothesis, I found that pharmacological inhibition of prelimbic D1 dopamine receptors disrupted the locomotor activating effects of the 1.5 mg/kg dose of amphetamine to a greater extent in adolescent than in adult rats. In addition, the stimulation of prelimbic D1 dopamine receptors potentiated locomotor activity at the 0.5 mg/kg dose of amphetamine only in adolescent rats, indicating that the prelimbic D1 dopamine receptors are involved in overcoming locomotor hypoactivity during adolescence.

Given my finding that the locomotor activating effects of amphetamine rely on slightly different mechanisms in adolescence than in adulthood, study 4 was designed to determine whether the lasting consequences of drug use would also differ with age. A short period of pre-treatment with 0.5 mg/kg of amphetamine in adolescence, but not in adulthood, resulted in heightened sensitivity to an injection of amphetamine given 30 days after the start of the procedure, when adolescent rats had reached adulthood. The finding of an age-specific increase in amphetamine sensitivity is consistent with evidence for increased risk for addiction when drug use is initiated in adolescence compared to

adulthood in people (Merline et al., 2002), and with the hypothesis that adolescence is a sensitive period of development.

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CHAPTER 1: GENERAL INTRODUCTION

Drug use and adolescence

Adolescence is a period of development during which individuals are driven to explore novel stimuli, as evidenced by high levels of sensation-seeking and risk taking during this period of development (reviewed in Spear, 2000 Steinberg, 2004). The drive to explore often extends to drugs of abuse to such an extent that experimentation with drugs in adolescence may be considered normative (Spear, 2000). However, a lot of evidence suggests that the consequences of drug use in adolescence are more severe than are the consequences of drug use in adulthood. For example, adolescents progress from drug use to dependence more rapidly than adults (Clark, Kirisci, & Tarter, 1998), and adults who initiated drug use in adolescence suffer from more serious dependence problems than those who initiated drug use later in life (e.g., Clark et al., 1998; Merline, O'Malley, Schulenberg, Bachman, & Johnston, 2004; Windle, Spear, Fuligni, Angold, & Brown, 2008). Psychostimulants, including cocaine and methamphetamine, represent the greatest abuse problem in the United States (Vetulani, 2001) and a growing concern in Canada. According to Health Canada, cocaine and amphetamine use has been on the rise since 1994, in large part because of increasing drug use in adolescence (Adlaf, Begin, & Sawka, 2005). Amphetamine in particular is abused world-wide because of its cheaper cost and longer lasting effects compared to cocaine (Klee, 1992; Murray, 1998; Selden, 1991).

Animal models of drug use and addiction

Animal models are essential for developing an understanding of drug-responsive neural circuits and for mapping cause and effect relationships between environmental

manipulations, development, and propensity for drug abuse. For findings from animal models to be translated to people, animal models must meet the criteria for face, construct, and predictive validity (Sanchis-Segura & Spanagel, 2006; Schramm-Sapyta, 2004; Spear, 2000). In the rodent model of drug abuse, face validity is evaluated based on similarities in drug-taking behaviour between people and rodents, whereas predictive validity is evaluated by the ability of drug responses in rodents to predict responses in people. Both criteria are met by the fact that when given the opportunity, rodents self-administer and avoid the same drugs that people do, such that drug use in rodents can successfully predict the abuse potential of drugs in people (Koob, 2000; Sanchis-Segura & Spanagel, 2006). Finally, construct validity is assessed based on overlapping drug-responsive neural circuitry, which is remarkably similar in people and in rodents (Schramm-Sapyta, 2004).

The main goal of research in animal models is to identify the risk factors that increase the probability of drug abuse and addiction (Sanchis-Segura & Spanagel, 2006). Drug abuse is defined as the improper use of legal or illegal drugs for the purpose of altering mood or consciousness (Cuddy, 2003), whereas addiction is a disorder of the central nervous system, characterized by compulsive drug use, craving and drug seeking in spite of negative consequences and at the expense of personal relationships (Cuddy, 2003; Schramm-Sapyta, 2004; Vetulani, 2001). Addiction develops over a long period of drug intake, which in rats may require at least 3 months of drug exposure (Deroche-Gamonet, Belin, & Piazza, 2004). For this reason, most animal work has focused on modeling specific behavioural components that lead to addiction, rather than the culmination of these components in addiction (Sanchis-Segura & Spanagel, 2006).

Specifically, individual differences in the rewarding and stimulating effects of drugs in the early stages of drug use, as well as individual differences in adaptations to repeated drug use, are associated with different levels of addiction risk (Haertzen, 1986; Haertzen, Kocher, & Miyasato, 1986; Robinson & Berridge, 1993; Robinson & Berridge, 2000). For this reason, many studies focus on individual, environmental, and developmental factors that are involved in altering the reinforcing and stimulating effects of drugs.

An additional goal of research with animal models is to use pharmacological tools to investigate the functions of different neurotransmitter systems and to map functions of specific neural regions and circuits. Different drugs of abuse elicit effects on behaviour by altering distinct aspects of neurochemical balance (Lüscher & Ungless, 2006), which makes them useful for investigating neural responses to pharmacological stimulation or inhibition of various neurotransmitter systems. Drugs can also be used to identify drug-responsive neural regions by injecting them directly into specific sites in the brain through surgically implanted cannulae. Comparisons of behavioural outcomes produced by these injections are then used to identify the relative contribution of different regions in production of a single behavioural outcome, or to identify distinct roles for different regions in distinct behavioural outcomes (Ikemoto & Wise, 2004). The latter approach has been particularly useful for identifying the neural substrates that are involved in drug addiction, based on evidence that all addictive drugs, irrespective of their primary mechanism of action, involve actions on common neural substrates, most notably the mesocorticolimbic dopamine system (discussed below) (Nestler, 2005).

Animal models of drug reward

Drugs are positive reinforcers that elicit quantifiable approach behaviours in rodents (Koob, 2000; Robinson & Berridge, 2000; Shippenberg & Koob, 2002). Drug-seeking can be defined as an increase in the rate of drug consumption with repeated administrations, or as the development of a preference for the drug. The most commonly used measures of reinforcing value of drugs in rodents include self-administration and conditioned place preference (CPP). In self-administration paradigms, animals are implanted with a drug-delivery device that they can learn to control by performing an instrumental action. Drug delivery can occur on a fixed schedule, whereby the drug is administered after a fixed number of instrumental actions (e.g., lever presses) have occurred, or on a progressive ratio schedule, whereby the number of instrumental actions required to obtain the drug increases progressively throughout the procedure. Both procedures provide information about the animal's desire (wanting) to consume the drug, and the latter allows for evaluation of the animal's motivation to work for the drug as the cost of obtaining the drug increases (Sanchis-Segura & Spanagel, 2006).

Conditioned place preference (CPP) is a classical conditioning procedure that has been argued to measure a variety of reinforcing properties of drugs, including their hedonic value, drug-seeking behaviour, drug liking, and drug wanting (Bardo & Bevins, 2000; Shippenberg & Koob, 2002). CPP involves the repeated pairing of a drug with a specific environmental cue and pairing of saline (i.e., vehicle) with another cue. The reinforcement value of the drug is measured by the difference in time spent on the drug-paired cue relative to the saline-paired cue (Bardo & Bevins, 2000). Conversely, an animal is considered to have developed aversion to the drug when they spend more time

on the saline compared to the drug-paired cue. Validity of this tool is supported by evidence that rodents develop a preference for drugs that are reinforcing in people and an aversion for drugs that have known negative effects in people (Shippenberg & Koob, 2002).

Locomotor activating effects of psychostimulants

In addition to producing reinforcing effects, psychostimulants increase locomotor activity in rodents. Locomotor activating and reinforcing effects of psychostimulants are closely associated, as demonstrated by their reliance on similar neural circuitry (e.g., Robinson & Berridge, 1993; Robinson & Berridge, 2000; Smith, Tindell, Aldridge, & Berridge, 2009; Wise & Bozarth, 1987). This relationship is thought to exist because reinforcing stimuli must elicit approach behaviours in order for organisms to interact with and obtain the reinforcing stimulus (Mogenson, Jones, & Yim, 1980). Essentially, locomotor activity may be considered as an easily measured component of translating motivation into action (Mogenson et al., 1980), which has made this measure a widely used index of psychostimulant actions in the central nervous system.

Locomotor sensitization

Repeated, intermittent administration of psychostimulants results in locomotor sensitization, which is defined as the progressive augmentation of locomotor activity with successive drug treatments (e.g., Kuczenski, Segal, & Todd, 1997; Lett, 1989; Lorrain, Arnold, & Vezina, 2000; Robinson & Berridge, 2000; Shippenberg & Koob, 2002). Although sensitization is most commonly assessed with respect to locomotor activity, this phenomenon has also been reported for the reinforcing effects of drugs. For example, amphetamine pre-treated rats worked harder to self-administer amphetamine compared to

saline pre-treated rats on a progressive-ratio schedule of reinforcement (Vezina, Lorrain, Arnold, Austin, & Suto, 2002). These data suggest that amphetamine pre-treatment increases the sensitivity to amphetamine's subsequent reinforcing effects and reduces the sensitivity to the increasing cost associated with the high number of lever presses required to obtain the drug (Vezina et al., 2002). This increase in sensitivity to the locomotor activating and reinforcing effects of drugs is thought to reflect the neural processes involved in transition from drug abuse to addiction (Robinson & Berridge, 1993; Robinson & Berridge, 2000), which has made locomotor sensitization a commonly used measure of addiction risk in rats.

Drug-responsive neural circuitry

All addictive drugs, including psychostimulants like amphetamine, increase dopamine activity in the mesolimbic dopamine system, which is thought to be the basis of their addictive properties (Nestler, 2005; Pierce & Kumaresan, 2006). Amphetamine is a dopamine releaser and an uptake inhibitor that increases levels of the neurotransmitter dopamine in the synapse (Feldman, Meyer, & Quenzer, 1997). Although amphetamine can enter the cell through passive diffusion, the majority of its effects are produced through interactions with the dopamine transporter on the presynaptic cell. Under normal conditions, dopamine in the synapse is transported back into the cell by binding to its transporter, thereby reducing the amount of transmitter in the extracellular space. When amphetamine binds to the dopamine transporter, it causes it to reverse direction, thereby moving dopamine out of the synaptic terminal (Leshner & Koob, 1998; Seiden, Sabol, & Ricuarte, 1993). This process is referred to as reverse transport and it results in dopamine overflow throughout neural regions that contain the dopamine transporter, including the

nucleus accumbens (Sellings & Clarke, 2003; Vezina, 2004), prefrontal cortex (Shoblock, Maisonneuve, & Glick, 2004), and the ventral tegmental area (Adell & Artigas, 2004). This overflow has been implicated in reinforcing and locomotor activating effects of amphetamine (Lorrain, Riolo, Matuszewich, & Hull, 1999; Vezina, 2004).

Dose and route of administration

Effects of amphetamine on behaviour are dose-dependent. In most laboratory studies, systemic administrations of amphetamine are given intraperitonealy (i.p.) into the body cavity, although subcutaneous injections are also used. With these routes of administration, amphetamine doses of less than 0.1 mg/kg do not produce an observable effect on behaviour. Doses between 0.1 and 0.4 mg/kg represent the ED_{50} (an effective dose that produces a response in 50% of rats) range and generally induce an increase in locomotor activity. Doses between 0.4 to 1.0 mg/kg reliably increase locomotor activity in adult rats and intensity of locomotion is increased with high doses (1.0 to 3.0 mg/kg). Doses greater than 3.0 mg/kg are considered to be very high, such that locomotor activity is suppressed and is replaced by stereotypy (Grilly & Loveland, 2001; Rebec, White, & Puotz, 1997). Stereotypy refers to a set of repetitive behaviours, such as head bobbing and sniffing, which normally occur at a single location in the apparatus, and may be more closely associated with psychosis and aversion than with reward (Adriani, Chiarotti, & Laviola, 1998; Robinson & Becker, 1986).

The mesocorticolimbic dopamine system refers to a group of related structures that are innervated by dopaminergic projections from the ventral tegmental area. The ventral tegmental area contains dopamine cell bodies with axons terminating in the nucleus accumbens (Feldman et al., 1997; Gold, Geyer, & Koob, 1989; Vezina, 2004),

the amygdala (Fallon, 1988), the hippocampus (Swanson, 1982) and the prefrontal cortex (Leshner & Koob, 1998). The effects of psychostimulants are initiated through actions in these brain regions, which regulate the expression and the intensity of reinforcing and locomotor activating effects of psychostimulants (Pierce & Kalivas, 1997).

The nucleus accumbens is directly implicated in the reinforcing and locomotor activating effects of psychostimulants. Lesions of dopamine terminals in the nucleus accumbens block psychostimulant-induced locomotor activity (Joyce & Koob, 1981; Sellings & Clarke, 2003), self-administration (Koob, 2000), and conditioned place preference (Sellings & Clarke, 2003). Conversely, amphetamine is readily self-administered directly into the nucleus accumbens (Phillips, Robbins, & Everitt, 1994) and administration of amphetamine into the nucleus accumbens produces a robust locomotor response (e.g., Fletcher, Korth, Sabijan, & DeSousa, 1998). These effects of amphetamine are blocked by administration of dopamine receptor antagonists (Phillips et al., 1994) and are mimicked by administration of dopamine receptor agonists (e.g., Meyer, Van Hartesveldt, & Potter, 1993) into the nucleus accumbens, indicating that locomotor activating and reinforcing effects of amphetamine occur through amphetamine-induced enhancement of dopamine release in this brain region. Given its role in the locomotor activating and reinforcing effects of psychostimulants, the nucleus accumbens is thought to provide a functional link between limbic and motor systems, which is required for translation of motivation into action (Mogenson et al., 1980).

Although the locomotor activating and reinforcing effects of amphetamine exhibit extensive overlap, there is evidence for specialization of different subregions of the nucleus accumbens for each behaviour (e.g., Budygin et al., 2004; Phillips, Setzu, &

Hitchott, 2003). The dorsal portion of the nucleus accumbens is referred to as the core, and the ventral portion as the shell, which can be further divided into ventral and medial portions (Feldman et al., 1997; Ikemoto, Qin, & Liu, 2005). Lesions of dopamine terminals in the nucleus accumbens core blocked the expression of psychostimulant-induced locomotor activity, but not conditioned place preference, whereas lesions in the shell blocked conditioned place preference and not locomotor activity (Sellings & Clarke, 2003). Similarly, rats learned to self-administer amphetamine into the medial shell of the nucleus accumbens, but not into the ventral shell or the core (Ikemoto et al., 2005). Thus, while locomotion and reward do co-occur and are generally predictive of one another, they can be localized to different, though proximal, regions in the brain. Furthermore, the proximity of these brain areas and their concurrent activation in the absence of surgical intervention support the view that locomotion and reward are closely related systems that function together to reinforce and drive behaviour.

Other components of the mesocorticolimbic dopamine system are not directly involved in the initiation of behavioural responses to psychostimulants, although they do mediate these responses. For example, administration of amphetamine directly into the prefrontal cortex or the ventral tegmental area did not affect locomotor activity, but injections of amphetamine into these regions altered the locomotor activating effects of acute or repeated injections of systemically administered amphetamine (Kalivas & Webber, 1988; Lacroix, Broersen, Feldon, & Weiner, 2000). Similarly, amygdala lesions enhanced locomotor activating effects of systemically administered amphetamine although lesions alone had no effect on basal activity (Woods & Ettenberg, 2004). Thus, although amphetamine-induced locomotor activity is initiated in the nucleus accumbens,

the magnitude of the locomotor response is modulated by other components of the mesocorticolimbic dopamine system.

Dopamine receptors

Dopamine binds to cell-surface receptors that are coupled to transmembrane G proteins, which allow the dopamine-receptor complex to alter levels of second messengers inside the cell. Association of dopamine with its receptor leads to activation of adenylate cyclase inside the cell, which catalyzes the conversion of adenosine triphosphate (ATP) into the second messenger cyclic adenosine monophosphate (cAMP). Accumulation of cAMP leads to the activation of protein kinase A (PKA), which regulates the active state of downstream effectors through phosphorylation of serine and threonine residues. One of the best understood targets of this signaling cascade is the cAMP response element binding (CREB) protein, which is a constitutively expressed transcription factor that becomes active when phosphorylated at serine 133 by protein kinase A (Herdegen & Leah, 1998). Once phosphorylated, CREB goes on to regulate the expression of genes associated with synaptic plasticity (Barco, Jancic, & Kandel, 2008). Phosphorylated CREB (pCREB) has received much attention in studies of addiction because of the ability of psychostimulants to induce CREB phosphorylation throughout the mesocorticolimbic dopamine system (McGinty, Shi, Schwendt, Saylor, & Toda, 2008), which also makes pCREB a useful measure of psychostimulant-induced activation of neural regions.

There are five subtypes of dopamine receptors that can be classified on the basis of their effects on cAMP synthesis as belonging to either the D1-like or the D2-like dopamine receptor family (Moreira et al., 2010; Nichols, 2010). D1-like receptors are

comprised of the D1 and D5 receptor subtypes and D2-like receptors are comprised of the D2, D3, and D4 receptor subtypes. The major distinction between the two classes of dopamine receptors is their opposing effect on cAMP, whereby D1-like receptors increase, and D2-like receptors decrease, cAMP accumulation in the cell (Neve, Seamans, & Trantham-Davidson, 2004; Romanelli, Williams, & Neve, 2010). Different actions of these receptors on cAMP are attributed to coupling to different G proteins. Specifically, D1 receptors are coupled to excitatory G proteins ($G_{\alpha s}$\$G_{\alpha olf}$) that stimulate adenylate cyclase, whereas D2 receptors activate inhibitory G proteins ($G_{\alpha i}$) that inhibit adenylate cyclase and thus prevent the conversion of ATP to cAMP (Moreira et al., 2010). Although the cAMP-PKA cascade is most strongly associated with dopamine signaling, dopamine receptors also have effects on other signaling pathways, including the phosphoinositide and the mitogen-activated protein kinase cascades (Jin, Cai, Wang, Smith, & Friedman, 1998; Romanelli et al., 2010). Moreover, dopamine receptors can also regulate neurotransmission through phosphorylation of ion channels, as well as glutamate and GABA receptor subunits (reviewed in Romanelli et al., 2010).

Role of dopamine receptors in the locomotor activating and reinforcing effects of amphetamine

Functional roles of dopamine receptors are classically assessed with administration of dopamine receptor agonists and antagonists systemically or directly into the brain. Agonists are drugs that mimic effects of endogenous ligands and antagonists are drugs that antagonize or block the effects of endogenous ligands at the receptor(s) of interest. Indirect dopamine receptor agonists such as amphetamine increase the amount of dopamine available in the synapse, which leads to non-specific activation of dopamine

receptors (Romanelli et al., 2010). For this reason, amphetamine and other indirect dopamine receptor agonists can be used to investigate the overall sensitivity of the central nervous system or of specific brain sites to dopamine stimulation, but no conclusions can be made about the role of specific receptors. For this reason, direct dopamine receptor agonists and antagonists have been developed that bind selectively to specific dopamine receptor classes or subtypes. Currently, no drugs are available that can distinguish between D1 and D5 receptor subtypes, although many drugs have been developed that selectively bind to D1-like receptor families (Nichols, 2010). For example, SCH 23390 is the first highly selective D1-like receptor antagonist (Bourne, 2001) and SKF 81298 is a highly effective D1-like receptor agonist (Desai, Terry, & Katz, 2005). A number of D2 receptor agonists and antagonists also exist, and many of these are selective for specific receptor subtypes (Prante, Dörfler, & Gmeiner, 2010), although some widely used drugs, like raclopride, act as potent antagonists at multiple D2-like receptor subtypes (Köhler, Hall, Ögren, & Gawell, 1985). These drugs have been widely used to investigate the function of different dopamine receptors in the central nervous system.

Dopamine D1 and D2 receptors are critical for the locomotor activating and reinforcing effects of psychostimulants. Administration of D1 or D2 receptor agonists directly into the nucleus accumbens increases locomotor activity and injection of D1 or D2 receptor antagonists inhibits locomotor activity (Dreher & Jackson, 1989; Meyer, 1993; Meyer et al., 1993) . There is a synergistic effect of D1 and D2 receptor agonists in the nucleus accumbens, such that co-administration of both agonists increased locomotor activity more than administration of either agonist alone (Dreher & Jackson, 1989). Moreover, injection of D1 agonists alone into the nucleus accumbens is more effective at

increasing locomotor activity than is an injection of D2 agonists alone (Dreher & Jackson, 1989). In addition, injections of either D1 or D2 receptor agonists directly into the nucleus accumbens effectively induce conditioned place preference (White, Packard, & Hiroi, 1991) and injections of either D1 or D2 receptor antagonists abolished amphetamine conditioned place preference (Liao, 2008).

There is also a role for D1 and D2 dopamine receptors in the medial prefrontal cortex, as injections of a D1 receptor antagonist (Hall, Powers, & Gulley, 2009) or a combined D1/D2 receptor antagonist (Bast, Pezze, & Feldon, 2002) into this brain region blocked locomotor activating effects of systemically administered amphetamine. Further, injections of D1 receptor agonists into the medial prefrontal cortex increased preference for a low dose of systemically administered cocaine, although D1 receptor injection alone did not produce a preference (Brenhouse, Sonntag, & Andersen, 2008). The latter findings are consistent with a role for dopamine receptors in the medial prefrontal cortex in regulating locomotor activating and reinforcing effects of psychostimulants.

Distribution of dopamine receptors in the mesocorticolimbic dopmaine system. An important source of functional differences in different dopamine receptor subtypes comes from differences in their distribution in the central nervous system (Romanelli et al., 2010). The highest density of D1 dopamine receptors is found in the nucleus accumbens, moderate density is found in the amygdala, and lowest density in the ventral tegmental area, the hippocampus and the medial prefrontal cortex (Dubois, Savasta, Curet, & Scatton, 1986; Savasta, Dubois, & Scatton, 1986; Wamsley, Gehlert, Filloux, & Dawson, 1989). High density of D2 dopamine receptors is also found in the nucleus accumbens and moderate density is found in the hippocampus, although density of D2

receptors is much lower compared to D1 receptors in the cerebral cortex (Wamsley et al., 1989). The high distribution of both D1 and D2 dopamine receptors in the nucleus accumbens compared to other neural sites is consistent with the primary role for dopamine receptors in modulation of motivated behaviour in this region.

Defining adolescence

Adolescence is a unique stage of development during which individuals must make the transition from being highly dependent on parents in childhood to becoming independent individuals with relationships outside of the home (Wahlstrom, Collins, White, & Lucian, 2010). As part of negotiating the successful transition into adulthood, adolescents place a high value on peer relationships outside of the home, they exhibit increased exploration of novel stimuli, and engage in higher levels of risk-taking and impulsive decision making compared to adults (Ernst, Romeo, & Andersen, 2009; Spear, 2000; Wahlstrom et al., 2010). Although this behavioural profile is often associated with negative outcomes, it is also thought to facilitate the successful transition into adulthood by allowing for exploration of adult roles (Silbereisen & Reitzle, 1992; Wahlstrom et al., 2010).

The drive to explore unfamiliar stimuli and to seek novel peer relationships is in part driven by an enhanced sensitivity to the rewarding, and reduced sensitivity to aversive, stimuli during adolescence (Ernst et al., 2009; Spear, 2000; Wahlstrom et al., 2010), which can place adolescents at an increased risk for drug abuse and addiction. In people, approximately half of all psychostimulant use is initiated in adolescence (Kandel & Logan, 1984; Wu & Schlenger, 2003), when rapid brain development alters vulnerability to addictive substances (Chambers, Taylor, & Potenza, 2003; Schramm-

Sapyta, 2004; Smith, 2003). Use of illicit drugs in adolescence is associated with greater severity of drug abuse problems in adulthood (Merline et al., 2004) and adolescents and young adults exhibit more rapid transition from drug use to dependence compared to adults (Spear, 2000; Wu & Schlenger, 2003). Nonetheless, there is far less research on the effects of psychostimulants in adolescence using animal models than in adulthood.

Rodent models of adolescence exhibit remarkable parallels to adolescence in people. As in people, adolescent rodents exhibit heightened levels of novelty seeking (Adriani et al., 1998; Smith, 2003; Stansfield & Kirstein, 2006; Stansfield, Philipot, & Kirstein, 2004), impulsivity (Laviola, Adriani, Terranova, & Gerra, 1999; Schramm-Sapyta, Walker, Caster, Levin, & Kuhn, 2009; Spear, 2000), and peer affiliation (Spear, 2000), as well as increased sensitivity to rewarding and decreased sensitivity to aversive stimuli (Badanich, Adler, & Kirstein, 2006; Brenhouse, Dumais, & Andersen, 2010; Brenhouse, Sonntag et al., 2008; Ernst et al., 2009; Infurna & Spear, 1979; Schramm-Sapyta, Morris, & Kuhn, 2006; Spear, 2000; Wahlstrom et al., 2010) compared to adults. These parallels between people and rodents demonstrate the usefulness of rodent models for investigation of age differences in risk for drug abuse and addiction.

Age span of adolescence

Adolescence lacks a clear onset and offset as a period of development in people and rodents alike (reviewed in McCormick & Mathews, 2007; Sisk & Foster, 2004). In people, the onset of adolescence is considered to begin at the onset of puberty, though many cognitive and social developments associated with adolescence continue thereafter (Sisk & Foster, 2004). Onset of adolescence is also defined by chronological age, usually marked by the beginning of teenage years in people (13 yrs of age) (Waylen & Wolke,

2004) and has been argued to conclude anywhere from 5-8 years following the onset of puberty (Rosenfeld & Nicodemus, 2003). A typical definition of adolescence in the rodent model is that of Tirelli and colleagues (Tirelli, Laviola, & Adriani, 2003), which divides adolescence into early- (postnatal day (P) 21; P21 - P34), mid- (P34 - P45), and late- (P45 - P59) stages. Most rodent research using adolescents to date has focused on the early stage, often concluding around "puberty", which begins at approximately 40 days of age in males and 35 days of age in females based on the markers of the day of preputial separation in males and the day of vaginal opening in females (reviewed in McCormick & Mathews, 2007).

Animal models of drug use in adolescence

Adolescent rats differ in response to psychostimulant treatment from adults, with adolescents typically exhibiting a reduced locomotor response to initial (acute) amphetamine treatment (Adriani & Laviola, 2000; Bolanos, Glatt, & Jackson, 1998; Lanier & Isaacson, 1977; Mathews & McCormick, 2007). Age differences in response to amphetamine may at least in part depend on dose, with some studies finding that age differences in locomotor activity can be reduced with high doses of amphetamine ($\geq$ 2.5 mg/kg) and reversed with very high doses of amphetamine (> 5.0 mg/kg) (Adriani & Laviola, 2000; Bolanos et al., 1998). Studies with cocaine have produced mixed results, with some finding reduced (Frantz, O'Dell, & Parsons, 2006; Schramm-Sapyta, Pratt, & Winder, 2004) and others finding enhanced (Badanich, Maldonado, & Kirstein, 2008) locomotor activity in adolescents compared to adults. Irrespective of the direction of the effect, most studies find that adolescents and adults differ in psychostimulant-induced

locomotor activity, which suggests that underlying neural substrates are differentially sensitive to psychostimulant treatment during adolescence.

Studies of adolescence often do not discriminate between pre- and post-pubertal adolescent rats, although there is evidence to suggest that the behavioural response to psychostimulants may change over stages of adolescence. For example, post-pubertal (P44), but not pre-pubertal (P27) adolescent rats exhibited higher conditioned place preference for 10.0 mg/kg cocaine compared to adults (Brenhouse, Sonntag et al., 2008), whereas P35, and not P45, rats exhibited greater preference than adults for 5.0 mg/kg cocaine (Badanich et al., 2006). Stage of adolescence may also influence locomotor activating effects to psychostimulants, with some evidence finding that P34 rats did not respond to either 2.0 or 5.0 mg/kg doses of amphetamine, whereas P45 rats responded to both doses (Lanier & Isaacson, 1977). Differences between stages of adolescence were also found for cocaine-induced locomotor activity, with P35, and not in P45, exhibiting higher levels of activity rats compared to adults (Badanich et al., 2006). Overall, these studies suggest that sensitivity to psychostimulants may change in a non-linear fashion over different stages of adolescence.

Adolescent rats also develop locomotor sensitization to repeated psychostimulant treatment more readily than adults (Adriani et al., 1998; Laviola et al., 1999; Mathews & McCormick, 2007; Schramm-Sapyta et al., 2004), indicating that the adolescent brain may be more readily shaped by psychostimulants. Indeed, treatment with psychostimulants in adolescence can induce locomotor sensitization that persists into adulthood (Achat-Mendes, Anderson, & Itzhak, 2003; Adriani et al., 2006; Brandon, Marinelli, Baker, & White, 2001; Burton, Nobrega, & Fletcher, 2010; Kolta, Scalzo, Ali,

& Holson, 1990; Marin, Cruz, & Planeta, 2008; McPherson & Lawrence, 2005; Ujike, Tsuchida, Akiyama, Fujiwara, & Kuroda, 1995), although it is not clear to what extent these effects are specific to adolescence, as most studies do not include an adult-pre-treated comparison group.

Adolescent development of the mesocorticolimbic dopamine system

These behavioural differences in psychostimulant responses between adolescents and adults most likely reflect ongoing development of the mesocorticolimbic dopamine system. The triadic theory of the neurobiology of motivated behaviour in adolescence (Ernst & Fudge, 2009; Ernst, Pine, & Hardin, 2005; Ernst et al., 2009) posits that age differences in behaviour are associated particularly with the development of, and connectivity between, three key regions: the nucleus accumbens, the amygdala, and the medial prefrontal cortex. These regions are of particular interest because they undergo extensive changes in adolescence, but also because their primary functions map on well to age differences in motivated behaviour during adolescence. According to the theory, the nucleus accumbens, the primary function of which is to mediate appetitive behaviour, is argued to be in a heightened state during adolescence, which biases adolescents to seek out rewarding stimuli. At the same time, the amygdala, the primary function of which is to mediate responses to aversive stimuli, is thought to be in a lowered functional state during adolescence, which makes adolescents less sensitive to negative stimuli. Finally, the medial prefrontal cortex, which weighs the inputs from these regions, is thought to be biased in favour of appetitive stimuli in adolescence. Thus, the increased engagement in risky behaviour, novelty seeking, drug use, and impulsive decision making during adolescence are thought to occur because of functional changes in these key regions,

although the relative contribution of each region in regulating behavioural responses in adolescence has not been directly investigated.

The structures outlined in the triadic theory are strongly interconnected. For example, the nucleus accumbens receives glutamatergic projections from the amygdala (Robinson & Beart, 1988) and from the medial prefrontal cortex (Porrino & Lyons, 2000), and the medial prefrontal cortex and the amygdala have dense reciprocal connections (Groenwegen, Wright, Beijer, & Voorn, 1999). Each of these regions and the connections between them are remodelled during adolescence. In the nucleus accumbens and the prefrontal cortex, D1 and D2 dopamine receptors are overproduced throughout early adolescence, reach peak levels between P28 and P40 and are subsequently pruned to adult levels, although pruning is protracted in the mPFC compared to the NAc (Andersen, Thompson, Rutstein, Hostetter, & Teicher, 2000; Gelbard, Teicher, Faedda, & Baldessarini, 1989; Giorgi et al., 1987; Tarazi & Baldessarini, 2000). In both regions, basal levels of cAMP are higher in adolescence (P40) than in adulthood and the ability of D1 and D2 dopamine receptors to regulate cAMP levels is reduced in adolescence (Andersen et al., 2000). In contrast, dopamine transporter density in the NAc reaches adult levels by P28, with no evidence of subsequent pruning (Galineau, Kodas, Guilloteau, Vilar, & Chalon, 2004). Dopaminergic projections from the ventral tegmental area to the medial prefrontal cortex continue to increase throughout adolescence and into young adulthood (>P60) (Berger, Verney, Febvret, Vigny, & Helle, 1985; Kalsbeek, Voorn, Buijs, Pool, & Uylings, 1988) and basal levels of dopamine in the nucleus accumbens increase between early and late adolescence (Badanich et al., 2006). In addition, dopaminergic regulation of electrophysiological properties of medial prefrontal

cortex neurons and of prefrontal inputs to the nucleus accumbens matures between adolescence and adulthood (Benoit-Marand & O'Donnell, 2008; Tseng & O'Donnell, 2007).

Morphological changes are also found in the medial prefrontal cortex and the amygdala in adolescence. Specifically, the volume of the prefrontal white matter increases between P30 and P90 in both sexes, whereas neuron number decreases in females more extensively than in males during the same period (Markham, Morris, & Juraska, 2007). The number of neurons and glia in the amygdala decreases from P35 to P90 (Rubinow & Juraska, 2009) and glutamatergic projections from the amygdala to the mPFC continue to increase until P60 (Cunningham, Bhattacharya, & Benes, 2002, 2008). Although the develoment of these regions likely contributes to age differences in response to psychostimulants, it is not clear how these developmental changes translate to age differences in behaviuor. Site-specific injections that are commonly used in adult rats have not been employed in the study of age differences in the sensitivity of individual components of the mesocorticolimbic dopamine system.

Puberty

Puberty is only one aspect of adolescence, as many developmental changes in the brain and behaviour occur independently of gonadal hormones, and behavioural and cognitive adaptations continue after puberty has passed (Pinyerd & Zipf, 2005; Spear, 2000). Puberty refers to the attainment of sexual maturity and is marked by increased pulsatility of the gonadotropin-releasing hormone (GnRH) and hypothalamic-pituitary-gonadal (HPG) axis activation (e.g., Payne, Kelch, Murono, & Kerlan, 1977; Wiemann, Clifton, & Steiner, 1989). GnRH is synthesized in the median eminence of the basal

hypothalamus and it signals the pituitary to release gonadotropins, including lutenizing hormone and follicle stimulating hormone, which direct the production of sperm, eggs, and steroid hormones from the testes and ovaries (reviewed in Sisk & Foster, 2004). Sixty days of age is generally thought to constitute adulthood, as physical and sexual maturity is achieved at this time (McCormick & Mathews, 2007; McCormick, Mathews, Thomas, & Waters, 2010).

Changes in hormone levels associated with adolescent maturation may be important for regulating at least some aspects of age differences in drug responses. Gonadal hormones are involved in regulating activity of the drug responsive mesocorticolimbic circuitry (Kuhn et al., 2010). Sex differences in drug responses emerge in adolescence, with females exhibiting higher levels of psychostimulant-induced locomotor activity and greater sensitivity to the reinforcing effects of psychostimulants than males (Becker, Molenda, & Hummer, 2001; Kuhn, Walker, Kaplan, & Li, 2001; Parylak, Caster, Walker, & Kuhn, 2008). Moreover, sex hormones mediate rewarding effects of psychostimulants (Walf, Rhodes, Meade, Harney, & Frye, 2007), although estrogen appears to be more effective than testosterone at regulating drug responses (Becker et al., 2001). The extent to which changes in gonadal hormones during puberty alter sensitivity to psychostimulants is less clear, but some evidence suggests that prepubertal gonadectomy alters the sensitivity to drug responses in adulthood (Forgie & Stewart, 1994a; Parylak et al., 2008).

Goal of the dissertation

Studies of adolescence in rodents have only recently gained momentum and much remains unknown about the factors that alter sensitivity to psychostimulant responses in

adolescence. Much of the available research is focused on understanding age differences in adaptations to prolonged periods of psychostimulant treatment, with relatively few studies focusing on age differences in the initial response to amphetamine. Understanding age differences in response to acute amphetamine is crucial for understanding the basis for altered sensitivity to drug abuse in adolescence. For example, studies in adult rats have found that a single injection of a high dose of amphetamine is sufficient for inducing lasting locomotor sensitization (Vetulani, 2001), indicating that a single dose of amphetamine can have profound effects on subsequent drug responses. Given that adolescents appear to be more susceptible to developing locomotor sensitization than are adults (Adriani et al., 1998; Laviola et al., 1999; Mathews & McCormick, 2007; Schramm-Sapyta et al., 2004), an understanding of age differences in response to acute amphetamine treatment is of critical importance for understanding the basis for age differences in sensitivity to immediate and lasting effects of psychostimulants.

Most studies of age differences in amphetamine responses that have been conducted thus far have used high doses ($\geq$ 2.0 mg/kg) (Adriani et al., 1998; Lanier & Isaacson, 1977; Niculescu, Ehrlich, & Unterwald, 2005) that are more closely associated with stereotypy than with locomotor activity (Grilly & Loveland, 2001). In contrast to locomotor activity, stereotypy is not dependent on the nucleus accumbens (Rebec et al., 1997), such that the use of high amphetamine doses may limit conclusions that can be drawn regarding functional differences in the primary region associated with reinforcing effects of psychostimulants. Further, most available studies are limited to a single stage of adolescence, with a large number of studies investigating pre-pubertal adolescent rats,

such that little is known about the extent to which neural and hormonal changes over different stages of adolescence contribute to age differences in drug responding.

The aim of this dissertation was to investigate age differences in locomotor activating effects of acute amphetamine in the dose range (0.5 and 1.5 mg/kg) that is associated with a dose-dependent increase in locomotor activity, while having a minimal impact on stereotypy (Grilly & Loveland, 2001). The goal of study 1 (chapter 2) was to conduct comparisons of locomotor activating effects of amphetamine in pre- (P30) and post- (P45) pubertal adolescent and adult rats in the same experiment to determine whether sensitivity to amphetamine changes over different stages of adolescence. In study 1, we also included comparisons of male and female rats to determine whether age differences in drug-responses are influenced by sex, as most studies of adolescence have only included male rodents. In adult rats, females are more sensitive to the locomotor activating and reinforcing effects of psychostimulants than are males, in part because of the influence of ovarian hormones (Becker et al., 2001). Thus, we investigated the potential role of gonadal horomones in regulation of age differences locomotor-activating effects of amphetamine in females that were ovariectomized before or after puberty, or in adulthood. Finally, we investigated developmental differences in tyrosine hydroxylase, a rate limiting enzyme in dopamine synthesis, as a marker of age differences in dopamine levels in the nucleus accumbens and the medial prefrontal cortex of rats.

Given that the nucleus accumbens is directly involved in locomotor activating effects of amphetamine and that this region undergoes extensive remodelling in adolescence, study 2 (chapter 3) was designed to investigate age differences in the locomotor activating effects of amphetamine produced by injections directly into the

nucleus accumbens. Having established that the sensitivity of the nucleus accumbens to the locomotor activating effects of amphetamine differs for adolescent and adult rats in study 2, study 3 (chapter 4) was designed to investigate the age differences in neural activation of the nucleus accumbens and the developing medial prefrontal cortex in response to the same doses of systemically administered amphetamine as in study 1 (0.5 and 1.5 mg/kg). Specifically, in study 3, age differences in amphetamine-induced activation of the nucleus accumbens and the medial prefrontal cortex were investigated using CREB phosphorylation and the expression of the immediate early gene c-fos as markers of neuronal activation. Having established that amphetamine produces different patterns of neural activation in the medial prefrontal cortex of adolescent and adult rats, further experiments were conducted to investigate age differences in the functional contribution of D1 dopamine receptors in the medial prefrontal cortex to locomotor activating effects of systemically administered amphetamine using injections of dopamine receptor agonists and antagonists directly into this region.

Finally, to determine whether age differences observed in the acute effects of low-dose amphetamine treatment would result in different consequences for adolescent and adult rats, rats in study 4 (chapter 5) were pre-treated with a low dose of amphetamine in adolescence or in adulthood and were tested for locomotor sensitization 30 days after the initial injection, when all rats were adult.

CHAPTER 2: CHANGES IN HYPORESPONSIVENESS TO ACUTE AMPHETAMINE AND AGE DIFFERENCES IN TYROSINE HYDROXYLASE IMMUNOREACTIVITY IN THE BRAIN OVER ADOLESCENCE IN MALE AND FEMALE RATS

Note: This section is based on the following article, with permission: Mathews, I.Z., Waters, P.G., & McCormick, C.M. (2009). Changes in hyporesponsiveness to acute amphetamine and age differences in tyrosine hydroxylase immunoreactivity in the brain over adolescence in male and female rats. *Developmental Psychobiology, 51,* 417-428.

Abstract

We investigated locomotor hyposensitivity after amphetamine in early (postnatal day 30; P30) and late (P45) adolescent rats compared to adults (P70) in experiment 1. Locomotor activity was measured for 1 hr after the first (acute) and second (24 hr later) injection of amphetamine (0.5 or 1.5 mg/kg). P30 and P45 rats were transiently hypoactive compared to adults, as indicated by reduced locomotor activity after acute amphetamine and enhanced activity after the second injection in adolescents only. In experiment 2, ovariectomy did not alter locomotor activity during habituation at any age compared to intact rats, and, as for intact adolescents, ovariectomized adolescents continued to be less active after amphetamine than adults, suggesting gonadal immaturity alone cannot account for age differences in experiment 1. However, ovariectomy attenuated the increase in activity after the second treatment. In experiment 3 involving untreated rats, tyrosine hydroxylase immunoreactivity was reduced in P30, P40, and P50 compared to P90 rats in the nucleus accumbens core and the medial prefrontal cortex. Thus, adolescents may have an increased threshold of behavioural activation that can be overcome with either a higher dose or with repeated amphetamine treatment, and may be related to changes in the dopamine system over development.

Introduction

In humans, there are age differences in sensitivity to drug effects that may underlie the increased risk for drug abuse and addiction in adolescents than in adults (Weiss, Mirin, & Bartel, 1994; Wu & Schlenger, 2003b). Adolescent and adult rodents also differ in sensitivity to the behavioural effects of acute and repeated psychostimulant treatment (reviewed in Izenwasser & French, 2002; Laviola, Macri, Morley-Fletcher, & Adriani, 2003; Spear, 2000; Tirelli, Laviola, & Adriani, 2003). Some studies have found that adolescent rats have reduced locomotor activity after an acute injection of amphetamine (Adriani & Laviola, 2000; Bolanos et al., 1998; Mathews & McCormick, 2007) or of cocaine (Frantz et al., 2006; Zombeck, Gupta, & Rhodes, 2009) compared to adults. Further, because adolescence is a transitional period covering the prepubertal and postpubertal time between weaning until sexual maturity (reviewed in McCormick & Mathews, 2007), sensitivity to amphetamine may also vary according to the age of adolescents under consideration. For example, male and female rats at postnatal Day 18 (P18) and in adulthood (> P60) increased locomotor activity after an acute injection of amphetamine (2.0, 5.0, or 10.0 mg/kg) compared to saline controls (Lanier & Isaacson, 1977). However, P45 rats required multiple treatments before an amphetamine induced increase in activity was observed and P34 rats did not respond at all (Lanier & Isaacson, 1977).

We previously reported that when adolescent and adult rats were compared directly using a lower range of doses of amphetamine (0.25, 0.5, or 1.0 mg/kg), P46 females, but not males, were less active after acute amphetamine than adults (Mathews & McCormick, 2007), even though these females were in late adolescence and were

postpubertal (i.e., cycling since approximately P35) at the time of testing. Moreover, only the adolescent females exhibited a significant increase in locomotor activity after a second injection of amphetamine 48 hr later, such that the age difference observed after the acute injection was no longer evident (Mathews & McCormick, 2007). Thus, locomotor hypoactivity in adolescent females may be a transient phenomenon that may reflect increased plasticity compared to adults. In the latter study (Mathews & McCormick, 2007), locomotor activity was recorded in small boxes during the training phase of the amphetamine conditioned place preference procedure, and we have also found hypoactivity to 1.0 mg/kg of amphetamine in male and female rats on P45 under more standard tests of locomotion with larger arenas (Mathews, Mills, & McCormick, 2008).

Thus, the first purpose of the present study was to determine whether acute hypoactivity that is limited to the first injection of amphetamine would be evident in both sexes in a more traditional test of locomotor activity [i.e., larger testing arena, longer test duration (60 instead of 30 min)], and using a higher dose of amphetamine (1.5 mg/kg) in addition to the 0.5 mg/kg dose we used previously. In addition, given the evidence that sensitivity to amphetamine changes across different adolescent ages (Lanier & Isaacson, 1977), we investigated the extent to which hypoactivity after amphetamine occurs in early adolescence in both males and females; thus, rats were tested either at P30, P45, or P70. Inclusion of different age groups in the same experiment also allowed for direct comparisons between rats at different adolescent ages to better characterize the trajectory of maturation of locomotor activity after amphetamine.

In a second experiment, we investigated the extent to which age differences in female rats were due to age differences in gonadal maturation, as sensitivity to psychostimulants is known to be regulated by estrogen in adult female rats (Festa & Quinones-Jenab, 2004; Forgie & Stewart, 1994a). This regulation likely develops in adolescence, as suggested by post-pubertal emergence of sex differences in sensitivity to cocaine (Kuhn et al., 2001; Parylak et al., 2008) and by altered cocaine (Parylak et al., 2008) and amphetamine (Forgie & Stewart, 1994b) sensitivity in adult female rats ovariectomized before puberty. Thus, to test whether differences in circulating ovarian hormones influence age differences in locomotor activity after amphetamine, we ovariectomized rats 6 days before amphetamine treatment either before puberty at P25, after puberty at P40, or in adulthood (P65).

Finally, because age differences in stimulant sensitivity have been attributed to the development of the mesocorticolimbic dopamine system (Chambers et al., 2003; Spear, 2000), and because adolescents and adults differ in baseline levels of extracellular dopamine in the nucleus accumbens (Badanich et al., 2006), we examined whether age differences in tyrosine hydroxylase, the rate limiting enzyme in the production of catecholamines, also differed across adolescence and into adulthood in untreated rats. Different levels of tyrosine hydroxylase in the nucleus accumbens have been reported for different strains of rats (Beitner-Johnson, Guitart, & Nestler, 1991; Gulley, Everett, & Zahniser, 2007) and for rats that differ in activity in a novel environment and after amphetamine treatment [apomorphine susceptible/unsusceptible rats: van der Elst, Roubos, Ellenbroek, Veening, & Cools, 2005; high/low responders: Verheij, de Moulder, De Leonibus, van Loo, & Cools, 2008], indicating that this enzyme may also be a good

marker for age differences in amphetamine sensitivity. Thus, in experiment 3, we measured tyrosine hydroxylase immunoreactivity in brain regions associated with the increase in locomotor activity after amphetamine, including the core of the nucleus accumbens (Sellings & Clarke, 2003) and the striatum (Rebec et al., 1997), as well as the medial prefrontal cortex, which has been implicated in regulation of activity in these brain regions (Pierce & Kalivas, 1997).

Methods

Animals

A total of 108 female and 64 male Long Evans rats from the Brock University colony were used in these experiments. The rats came from 20 different litters (20 primiparous dams, 6 sires). For breeding, pair-housed females were provided with a male for 5 days, after which the male was removed from the cage, and approximately 10 days later females were housed singly and provided with nesting material. Offspring were sexed and weighed on the first day of life (P0), and the litters were not culled (mean litter size of 14, range 10 to 18; mean number of males = 6.6, mean number of females = 7.4). The rats were weaned at 21 days of age (P21) and housed in groups of four (two per cage after Day 50) same-sex rats in a temperature controlled colony room with a 12:12 h light:dark cycle (lights on 08:00 h) with unlimited access to food and water. No more than one male and one female rat from a litter were assigned to any one experimental group. In experiment 1, male and female rats were tested at P30 (males: mean = 108.58 g, SD = 16.40, SEM = 4.73; females: mean = 98.67 g, SD = 16.88, SEM = 4.90), P45 (males: mean = 235 g, SD = 25.99, SEM = 7.50; females: mean = 185.75 g, SD = 23.81, SEM = 6.87) or P70 (males: mean = 455.5 g, SD = 37.89, SEM = 10.94; females: mean = 271.25 g, SD = 21.83, SEM = 6.30). In experiment 2, female rats were ovariectomized

(OVX) via bilateral flank incisions while under anesthesia (ketamine and xylazine mixture). Ovaries were extracted through a small incision (~5 mm) just below the ribs and the space below each ovary was clamped and sutured to prevent bleeding. The ovary was removed and remaining tissue was returned to the abdomen and the opening was closed with wound clips. Rats were allowed 5 days for recovery before habituation to the test arenas at P30, P45, or P70. Use of animals was approved by the Brock University IACUC and followed Canadian Council on Animal Care and National Institutes of Health guidelines.

Experiment 1

The test apparatus consisted of white open-top melamine boxes [58 cm (length); 58 cm (width); 58 cm (height)] with a melamine base. Four separate test arenas were used, and rats were always tested in the same arena. The arenas were cleaned after a test session with 50% ethanol. The arenas were illuminated indirectly by red light to reduce the suppressive effects of bright illumination on exploration. To habituate rats to the test conditions, on P30, P45, or P70, rats were injected with saline and then placed in the arenas for 30 min. In experiments 1 and 2, the first amphetamine (Sigma, UK) injection (0.5 or 1.5 mg/kg, i.p., dissolved in saline) was administered 24 hr after the habituation session (test Day 1; 1 h test session), followed by a second injection of the same dose 24 hr later (test Day 2; 1 h test session). Six rats were included in each age, sex, and dose group. Immediately after saline injection on habituation day and amphetamine injection on test days, locomotor activity was recorded using a Sony colour video camera mounted above the center of the boxes and linked to a computer tracking system (Smart; San Diego Instruments, San Diego, CA) that measured the distance traveled by the rat (cm).

We routinely find little change in locomotor activity after injection of saline over days of testing; the exception is when testing spans over a wide range of days in adolescence, there may be an increase in activity that is a reflection of development rather than simply an effect of repeated testing (Mathews & McCormick, 2007; Mathews et al., 2008; McCormick, Robarts, Gleason, & Kelsey, 2004; McCormick, Robarts, Kopeikina, & Kelsey, 2005). In addition, we reliably find increased locomotor activity in rats at the ages under consideration in the present experiments after amphetamine with a 0.5 mg/kg dose compared to saline treated rats, and more activity in amphetamine- than in saline-treated rats across test days (Mathews & McCormick, 2007). Therefore, to minimize the number of animals used, saline control groups were not included, and instead we relied on locomotion during habituation as a within-individual control.

Experiment 2

In experiment 2, 16 rats were OVX before puberty (P25), 15 after puberty (P40) and 15 in adulthood (P65). Rats were allowed 5 days to recover and were then tested as in experiment 1. A recovery period of 5 days was chosen so that rats could be OVX before and after puberty and still be tested for locomotor activity at the same ages as rats in experiment 1.

Experiment 3: Immunohistochemistry

Tyrosine hydroxylase immunoreactivity was measured in 26 female and 28 male rats at P30, P40, P50 and P90. These ages were chosen to represent early, mid, and late stages of adolescence, and adulthood, respectively. Rats were deeply anesthetized and transcardially perfused with physiological saline and 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were placed in a 30% sucrose and paraformaldehyde

solution until they sank. Coronal sections (40 μm thick) were collected throughout the medial prefrontal cortex [cingulate cortex area 1 and 2 (Cg1 and Cg2)], nucleus accumbens core, and the caudate nucleus using a cryostat (ThermoShandon) and stored in cryoprotectant at 80°C until the immunohistochemistry procedure could be performed. Every sixth section was collected for immunohistochemistry resulting in a total of four sections within the coordinates from approximately bregma 1.20 mm and 2.18 mm according to Paxinos and Watson (Paxinos & Watson, 2005). These coordinates were selected based on evidence that amphetamine increases activity most strongly after direct injection into the caudal-central region of the nucleus accumbens core (Essman, McGonigle, & Lucki, 1993).

Free floating sections were washed stringently in 0.1M PBS with 0.3% Triton X (PBSx, pH 7.1), then in 0.3% hydrogen peroxide, and then again in PBSx. Next, the tissues were incubated for 90 min in 10% normal horse serum (NHS) (Sigma) and then for 24 hr at 4°C in 1% NHS and tyrosine hydroxylase monoclonal primary antibody diluted at 1:10,000 (Sigma). After incubation, the sections were washed three times in PBSx before incubation for 75 min in biotinylated anti-mouse immunoglobulin secondary antibody (Vector Laboratories) diluted at 1:500 (Vector Laboratories). The sections were again washed in PBSx and placed for 1 hr in Avidin–Biotin Complex (Vector Laboratories). After another three washes in PBSx, tissues were placed in diaminobenzidine solution according to the instructions on the substrate kit (DAB SK-4100, Vector Laboratories) for 5 min. Immunostained sections were mounted onto slides, dried, and coverslipped with Permount. Sections were photographed at 400X magnification with a Nikon Eclipse brightfield microscope (see Figure 2.1 for regions in

which pictures were taken). TH-immunoreactivity (ir) was quantified using integrated optical density 3 SD above the background signal. Measures for each structure were averaged across hemispheres and sections, and animals for which there were fewer than four measures for a structure were excluded, resulting in final sample sizes of 6 ± 2 measures per animal for each brain region and for each sex and age group (n = 8 – 12 per group).

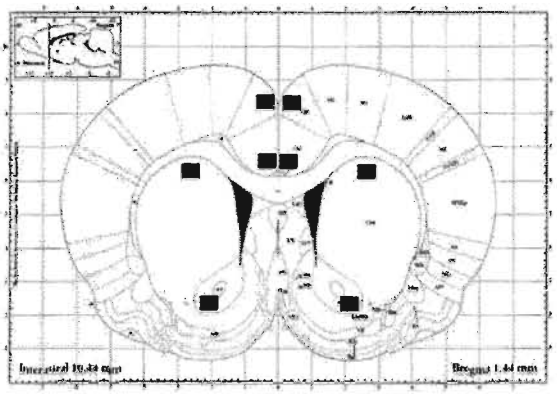

Figure 2.1. Regions used for the measurement of tyrosine hydroxylase immunoreactivity from the atlas of Paxinos and Watson (2005) as indicated by the black squares in the nucleus accumbens core, the caudate nucleus, and the medial prefrontal cortex (Cg1 and Cg2).

Statistics

Statistical analyses consisted of factorial and mixed factor analyses of variance. Sex was included as a between group factor in experiments 1 and 3 and Age was included as a factor in all experiments. Analyses in experiment 1 and 2 included the within-group factor of Days (1st and the 2nd amphetamine treatment day). Analyses were conducted for each dose separately. Main effects are not reported when they are obviated by interactions among effects. Similarly, two-way interactions are not reported when they are obviated by three-way interactions. Where appropriate, follow-up analyses were

conducted using F-tests for simple effects, paired t-tests and Fisher's protected least squares difference tests.

Results

Experiment 1

Habituation. There was an interaction of Sex and Age for distance traveled during habituation ($F(2,66) = 4.62$, $p = 0.01$). P30 females traveled less than P45 and P70 females (both $p < 0.0001$), and P45 and P70 females did not differ. P30 and P45 males traveled less than P70 males ($p = 0.01$ and 0.03, respectively), and P30 and P45 males did not differ. Females traveled longer distances than males at P45 ($p = 0.002$), but females and males did not differ significantly at P30 or P70 (see Figure 2.2 a).

Locomotor Activity after Amphetamine. For the 0.5 mg/ kg dose, there was an interaction of Sex and Age ($F(2,30) = 3.97$, $p = 0.03$) for locomotor activity over days of amphetamine treatment, whereby females traveled greater distances than males at P45 ($p = 0.005$) and P70 ($p = 0.002$), but not at P30. There also was an interaction of Age and Day ($F(2,30) = 4.00$, $p = 0.03$), whereby the increase in distance traveled from Day 1 to Day 2 was significant at P30 only ($p = 0.003$); on Day 1 ($F(2,33) = 18.54$, $p < 0.0001$), P30 rats traveled less than P45 ($p = 0.02$) and P70 ($p < 0.0001$) rats and P45 rats traveled less than P70 rats ($p < 0.001$); and on Day 2 ($F(2,33) = 11.31$, $p < 0.0001$), activity for P30 and P45 rats did not differ, and both traveled less than P70 rats (both $p < 0.0001$) (see Figure 2.2 b). For the 1.5 mg/kg dose, there was an interaction of Age and Day only ($F(2,30) = 4.69$, $p = 0.017$). Post hoc paired t-tests for sexes combined indicated that the increase in distance traveled from Day 1 to Day 2 was significant at P30 and P45 (both p

< 0.0001) and not at P70. However, the age groups did not differ on either day (see Figure 2.2 c).

To examine the extent to which the differences in locomotor activity after amphetamine were simply a reflection of age differences in locomotor activity, Sex X Age X Day ANOVAwere conducted on the percent increase in locomotor activity on Day 1 and Day 2 from activity during habituation (as a means of controlling for age differences and sex differences in general locomotor activity) for each dose separately. Sex was no longer a significant factor at any age, however the interaction of Age X Day remained significant for both doses (0.5 mg/ kg: $F(2,30) = 5.57$, $p = 0.009$, and 1.5 mg/kg: $F(2,30) = 6.38$, $p = 0.005$) (see Figure 2.2 d). For the 0.5 mg/kg dose, the difference in percent increase from habituation in distance traveled on Day 1 to Day 2 was significant at P30 only ($p = 0.004$); on Day 1 ($F(2,33) = 11.19$, $p < 0.0001$), P30 and P45 rats traveled less than P70 rats ($p < 0.0001$ and $p = 0.002$); and on Day 2 ($F(2,33) = 7.27$, $p = 0.002$), only the difference in activity between P45 and P70 rats was significant ($p = 0.001$). For the 1.5 mg/kg dose, the difference in percent increase from habituation in distance traveled on Day 1 to on Day 2 was significant only for P30 and at P45 rats (both $p < 0.0001$), and there were no significant age differences on either day.

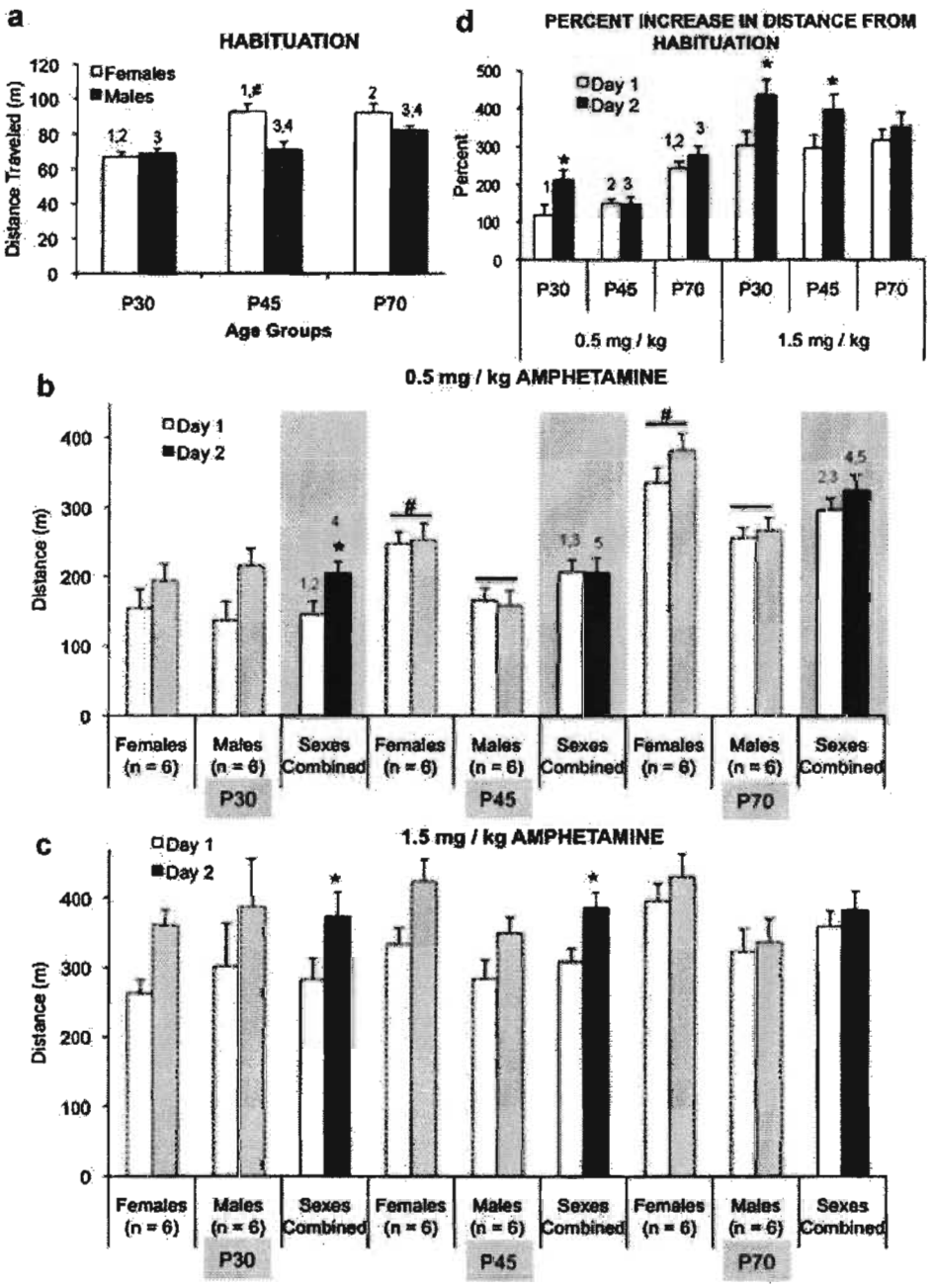

*Figure 2.2. Mean (±SEM) distance traveled in males and females and the sexes combined (shaded regions) in three different postnatal (P) age groups (a) on habituation day (interaction of Sex X Age); after injections of either (b) 0.5 mg/kg amphetamine (interaction of Sex X Age and Age X Day) or (c) 1.5 mg/kg amphetamine (interaction of Age X Day); (d) with distance traveled after amphetamine each day graphed as percent increase from habituation day (interaction of Age X Day was significant for both doses). Matched numbers indicate significant age differences for a test day; #a significant sex difference (females > males) for an age group; *a significant within-age difference over the two amphetamine days.*

Experiment 2

Habituation in Ovariectomized (OVX) Females. The main effect of Age was significant for locomotor activity during habituation ($F(2,43) = 21.33$, $p < 0.0001$): P30 rats traveled less than did P45 ($p = 0.001$) and P70 ($p < 0.0001$) rats, and P45 rats traveled less than did P70 rats ($p = 0.007$) (see Figure 2.3 a).

Locomotor Activity after Amphetamine in Ovariectomized (OVX) Females. An Age X Day ANOVA for distance traveled after 0.5 mg/kg of amphetamine obtained a main effect of Age ($F(2,22) = 58.95$, $p < 0.0001$): P30 rats traveled less than did P45 ($p = 0.001$) and P70 ($p < 0.0001$) rats, and P45 rats traveled less than did P70 rats ($p < 0.0001$) (see Figure 2.3 b). The ANOVA for distance traveled after 1.5 mg/kg of amphetamine also obtained an effect of Age ($F(2,18) = 6.61$, $p = 0.007$), such that P30 rats traveled less than P70 rats ($p < 0.0001$), and the distance traveled increased from Day 1 to Day 2 ($F(1,18) = 9.30$, $p = 0.007$) (see Figure 2.3 c).

To examine the extent to which the differences in locomotor activity after amphetamine were simply a reflection of age differences in locomotor activity, Age X Day ANOVA were conducted on the percent increase in locomotor activity on Day 1 and Day 2 from activity during habituation (see Figure 2.3 d). For the 0.5 mg/kg dose, the interaction of Age X Day was significant ($F(2,21) = 5.84$, $p = 0.009$), whereby the percent increase in distance traveled from habituation to test was lower on both Day 1 and Day 2 in P30 ($p = 0.006$, $p = 0.001$) and P45 ($p = 0.003$ and 0.047) compared to P70, and P70 had a higher percent increase on Day 2 compared to Day 1 ($p = 0.01$). For the 1.5 mg/kg dose, the effect of Day was significant ($F(2, 18) = 10.68$, $p = 0.004$), with greater percent increase in activity from baseline on Day 2 than on Day 1.

Additional post hoc analyses were conducted to compare the OVX rats in experiment 2 to the intact females in experiment 1.

P30 rats. Intact and OVX rats did not differ in distance traveled during habituation ($t(26) = 0.25$, $p = 0.81$). The Surgery X Day ANOVA for the 0.5 mg/kg dose of amphetamine revealed a significant interaction ($F(1,12) = 15.88$, $p = 0.002$). Activity increased from Day 1 to Day 2 in intact, but not in OVX females (see earlier analyses), such that OVX females were less active than intact rats on Day 2 ($p = 0.012$), but not on Day 1. The Surgery X Day interaction was also significant ($F(1,12) = 5.74$, $p = 0.03$) for the 1.5 mg/kg dose, but follow up analyses indicated that activity increased from Day 1 to Day 2 for intact and for OVX females (both $p < 0.05$). The effect of Surgery was not significant on Day 1 or on Day 2.

P45 rats. Intact and OVX rats did not differ in distance traveled during habituation ($t(25) = 0.92$, $p = 0.37$). The Surgery X Day ANOVA for the 0.5 mg/kg dose found a main effect of Surgery only ($F(1,12) = 10.85$, $p = 0.006$), such that OVX females were less active than intact females. At the 1.5 mg/kg dose, only the effect of Day was significant ($F(1,11) = 11.32$, $p = 0.006$), such that activity increased from Day 1 to Day 2 in OVX ($p = 0.02$) and in intact rats.

P70 rats. Intact and OVX rats did not differ in distance traveled during habituation ($t(24) = 1.79$, $p = 0.09$). The Surgery X Day ANOVA did not reveal any main effects or interaction at either dose.

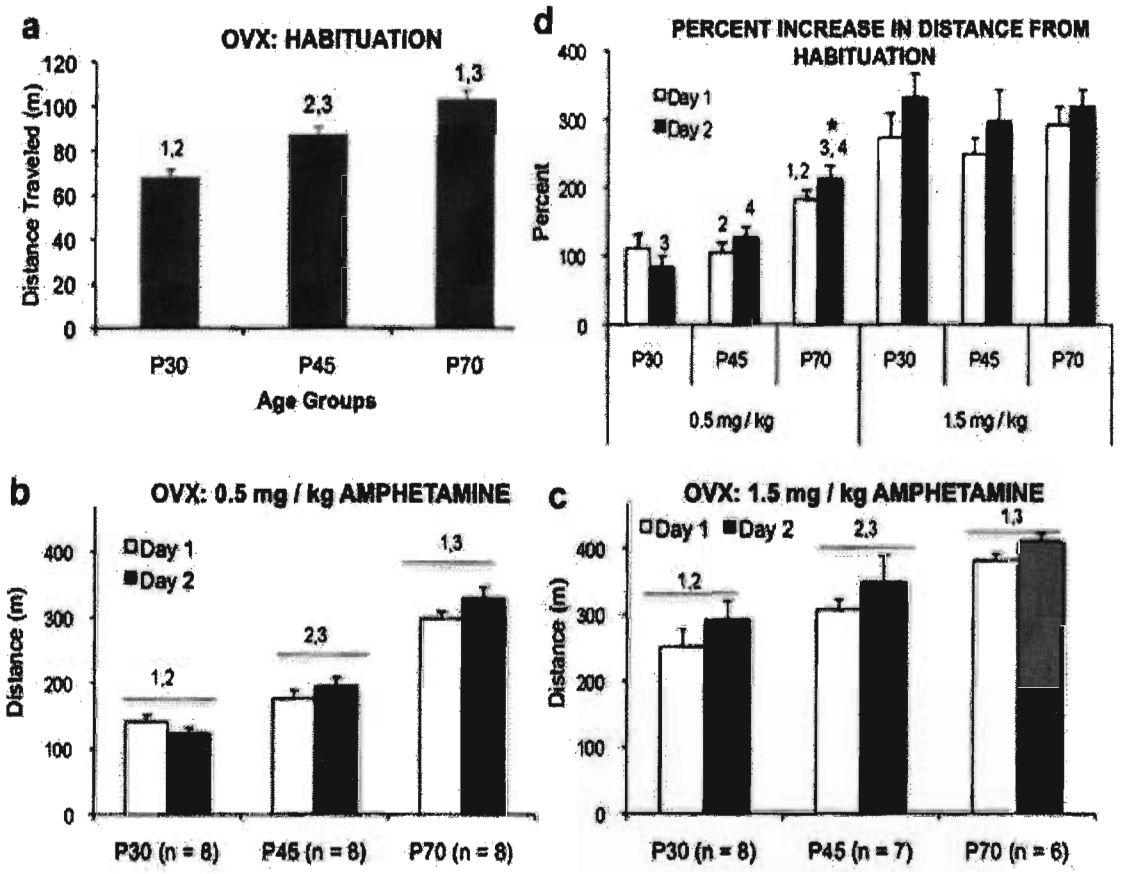

*Figure 2.3. Mean (±SEM) distance traveled in ovariectomized females in three different postnatal (P) age groups (a) on habituation day; after injections of either (b) 0.5 mg/kg amphetamine or (c) 1.5 mg/kg amphetamine; (d) with distance traveled after amphetamine each day graphed as percent increase from habituation day (significant interaction of Age X Day for 0.5 mg/kg, and significant main effect of Day for 1.5 mg/kg). Matched numbers indicate significant differences between age groups for a test day; *a significant within-age difference over the two amphetamine days.*

Experiment 3

Separate Age X Sex ANOVA was conducted for each of the four brain regions. The main effect of Sex and the interaction of Age and Sex was not significant for any region, whereas the main effect of Age was significant in the caudate ($F(3,44) = 9.13$, $p < 0.001$), the nucleus accumbens core ($F(3,37) = 4.17$, $p = 0.012$), Cg1 ($F(3,42) = 3.69$, $p = 0.019$), and Cg2 ($F(3,45) = 4.27$, $p = 0.01$). Post-hoc analyses indicated that in the caudate, P40 and P50 rats had reduced immunoreactivity compared to P30 ($p = 0.002$ for both) and P90 ($p = 0.002$ for both) rats, whereas P30 and P90 rats did not differ. In the nucleus accumbens core, P30 ($p = 0.009$), P40 ($p = 0.004$) and P50 ($p = 0.002$) rats had reduced immunoreactivity compared to P90 rats. In Cg1, immunoreactivity was reduced in P30 ($p = 0.02$) and P50 ($p = 0.003$) compared to P90 rats and in Cg2, P30 ($p = 0.003$), P40 ($p = 0.004$) and P50 ($p = 0.04$) rats had reduced immunoreactivity compared to P90 rats (see Figure 2.4).

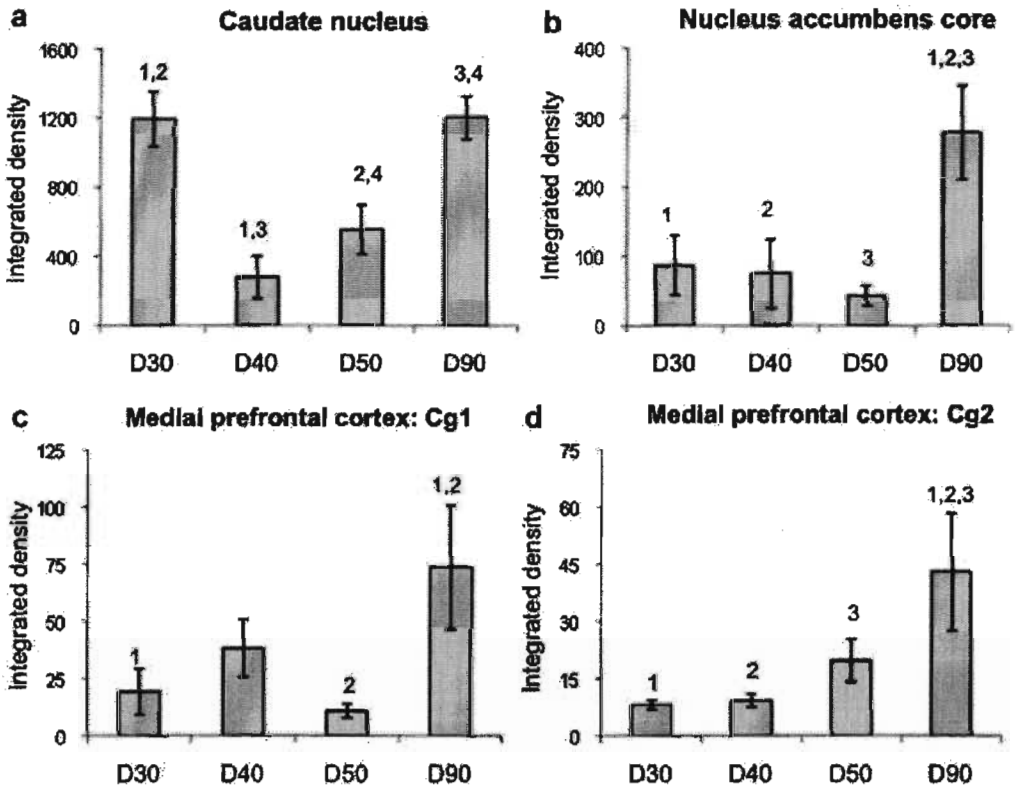

Figure 2.4. Mean (±SEM) tyrosine hydroxylase immunoreactivity (arbitrary units) in four different brain regions, (a) caudate nucleus, (b) nucleus accumbens core, (c) medial prefrontal cortex Cg1, and (d) medial prefrontal cortex Cg2, for separate postnatal (P) age groups. Matched numbers indicate significant differences between age groups. P30: n = 8, P40: n = 12; P50: n = 10; P90:n = 11.

Discussion

The main findings of the experiments above are that early and late adolescent males and females were hyporesponsive after acute amphetamine treatment compared to adults, and adolescents significantly increased activity to a second dose of amphetamine whereas adults did not. The initial hyporesponsiveness to amphetamine in adolescence does not appear to be due to gonadal immaturity based on the results in ovariectomized rats, but may be related in part to reduced catecholamine function, as indicated by lower tyrosine hydroxylase expression in adolescence than in adulthood in untreated rats. The specific findings are discussed below.

Locomotor activity after acute amphetamine treatment

The results from experiment 1 indicated that rats in early and in late adolescence are hypoactive after acute treatment with 0.5 or 1.5 mg/kg of amphetamine compared to adults. The initial hyporesponsiveness was not due to reduced locomotor capacity in adolescents, because, first, there were no age differences in locomotor activity to the second administration of the higher dose of amphetamine, and second, because the pattern of age differences in locomotor activity after the first dose of amphetamine did not parallel age differences during habituation. However, when locomotor activity was calculated as the percent change from habituation (to control for age differences in baseline activity), the difference between adolescents and adults remained only for the lower dose. Other studies investigating locomotor activity (Bolanos et al., 1998) and the acquisition of self administration (Shahbazi, Moffett, Williams, & Frantz, 2008) have also reported that age differences in rats are greater for low than for high doses of amphetamine. There also is evidence that the direction of age differences may reverse at

higher doses. Adriani and Laviola (Adriani & Laviola, 2000) found that male mice in mid-adolescence were less active than adults after 2.0 mg/kg and more active than adults after 10.0 mg/kg of amphetamine.

Another study found greater locomotor activating effects of acute amphetamine treatment only in adults with doses of 2.0 and 5.0 mg/kg and suppressed locomotor activity in adult, but not in adolescent rats with a 10 mg/kg dose (Lanier & Isaacson, 1977). Thus, there appear to be differences in the dose-response curve for amphetamine for adolescents and adults, with a decreased sensitivity manifested in adolescence to lower doses of amphetamine. However, there is a lack of consistency across studies as to the doses of amphetamine to which adolescents are hypoactive (Bolanos et al., 1998; Lanier & Isaacson, 1977; Mathews & McCormick, 2007), which may be due to testing conditions. A number of studies have shown that age differences in behavioral responses to psychostimulants depend on environmental and testing factors, including the size and shape of the testing arena, familiarity with the testing arena, test duration, and experimenter handling (amphetamine: Adriani & Laviola, 2000; amphetamine and cocaine: Bignami, 1996; cocaine: Frantz et al., 2006; cocaine: Schramm-Sapyta et al., 2004).

Change in activity to second administration of amphetamine

As predicted, we found that adolescent, but not adult rats, exhibited a significant increase in activity from the first to the second injection of amphetamine. For the 0.5 mg/kg dose, P30 rats increased locomotor activity after the second amphetamine treatment and P45 rats remained hypoactive, whereas both P30 and P45 rats increased activity after the second injection of 1.5 mg/kg of amphetamine. The fact that locomotor

activity increased after only one additional treatment with amphetamine in adolescent, but not in adult rats, has important implications, as it suggests locomotor hypoactivity after acute amphetamine in adolescence is accompanied by heightened plasticity to repeated treatment. The age difference in the increase in activity after a second dose of amphetamine remained when age differences in baseline activity were controlled. Thus, the extent to which adolescents are less sensitive (activity after acute amphetamine) or more sensitive (increase in activity after repeated amphetamine) depends on the measure. Similarly, Shahbazi et al. (Shahbazi et al., 2008) also showed that adolescent rats were less sensitive than adults on some measures of self-administration and more sensitive on others (for example, adolescents had increased drug intake and faster acquisition of self-administration than adults).

Hypoactivity after an acute injection of amphetamine that is not evident after a second injection suggests adolescents may have a higher threshold of activation than adults, but this threshold can be more easily shifted with repeated drug treatment or with a higher dose in adolescents than in adults. The neural basis of this behavioral profile is not known. Amphetamine increases locomotor activity by increasing dopamine release in the nucleus accumbens (Sharp, Zetterstrom, Ljungberg, & Ungerstedt, 1987; Vezina, 2004) and a single amphetamine treatment has been shown to induce different patterns of c-fos activation in this region in adolescent than in adult rats (Andersen, Leblanc, & Lyss, 2001). The decreased expression of tyrosine hydroxylase in adolescents than in adults found here suggests there may be reduced catecholamine pools available for release by amphetamine in adolescents than in adults. Consistent with the latter evidence, Laviola and colleagues (Laviola, Pascucci, & Pieretti, 2001) found that adolescent rats had lower

striatal dopamine release after acute amphetamine treatment compared to adults, but dopamine release was greater in adolescent than in adult rats that were repeatedly treated with amphetamine. Further, long term potentiation is enhanced in the nucleus accumbens during adolescence (Schramm, Egli, & Winder, 2002). Thus, enhanced drug-related change in locomotor behaviour is likely a reflection of greater plasticity in the mesolimbic circuitry in adolescence.

Locomotor activating effects of amphetamine: sex differences and the effect of ovarian hormones

Sex differences during habituation and in amphetamine induced locomotor activity were observed at P45 and P70 but not at P30 in the present study, such that females were more active than males in late adolescence and in adulthood (when females are post-pubertal, with estrous cycles beginning at approximately P35). Such a sex difference is well established in adult rats, and it has been attributed in large part to the potentiating effects of ovarian hormones in females (Becker et al., 2001), whereas only minimal inhibitory effects of testosterone have been reported for males (Becker et al., 2001). Studies with cocaine have also found that sex differences emerge after puberty (Kuhn et al., 2001; Parylak et al., 2008), and prepubertal ovariectomy has been shown to decrease the behavioral effects of cocaine (Kuhn et al., 2001) and amphetamine (Forgie & Stewart, 1994a) in adulthood. However, the effects of ovariectomy on drug responses in these studies were only examined in adulthood, so it is not clear whether lower circulating levels of ovarian hormones in adolescence contributed to age differences after amphetamine.

In experiment 2, there were no differences during habituation and few differences after amphetamine between intact and ovariectomized females at any age, suggesting the effects of ovariectomy are not attributable to the surgical procedure. The overall pattern of age differences in locomotor activity after amphetamine also did not change after ovariectomy, such that early and late adolescent females were still hypoactive compared to adults at both doses. As with intact rats, hypoactivity was no longer evident at the 1.5 mg/kg dose when age differences in locomotor activity during habituation were taken into consideration. However, ovariectomy did attenuate the overall locomotor activity and the increase in activity to the second injection of amphetamine compared to intact rats. This increase was entirely abolished in early adolescent females at the 0.5 mg/kg dose and attenuated in early and in late adolescent females at the 1.5 mg/kg dose. Thus, age differences in locomotor activity after amphetamine do not appear to be influenced by age differences in circulating ovarian hormones, though ovarian hormones may nonetheless contribute to plasticity after repeated amphetamine treatment in adolescence, even before puberty.

Gonadal hormones make important contributions to brain development and maturation over adolescence (Ahmed et al., 2008; Sisk & Foster, 2004). However, their contribution to development of the mesocorticolimbic dopamine system over adolescence may be minimal. For example, prepubertal gonadectomy had no effect on the transient increase in striatal dopamine receptor overexpression that occurs in adolescence and did not alter the pattern of sex differences in dopamine receptors (Andersen, Thompson, Krenzel, & Teicher, 2002).

Tyrosine Hydroxylase

Tyrosine hydroxylase immunoreactivity (TH-ir) was measured in untreated rats across adolescence (P30, P40, and P50) and in adulthood (P90) to determine whether catecholamine synthesis in regions associated with locomotor activating effects of amphetamine also changes in adolescence. Amphetamine enhances locomotor activity by increasing dopamine release in the nucleus accumbens core (Sellings & Clarke, 2003; Sharp et al., 1987; Vezina, 2004) and to a lesser extent in the caudate (e.g., Rebec et al., 1997), and activity of these regions is regulated in part by dopamine in the medial prefrontal cortex (Pierce & Kalivas, 1997).We found that in general, TH-ir in all of these regions was lower in adolescence than in adulthood.

TH-ir in the nucleus accumbens core was significantly lower in early, mid, and late stages of adolescence than in adulthood, but it is not clear how this may relate to extracellular levels of dopamine in this region. Frantz et al. (2006) found no age differences in baseline dopamine between adolescent and adult rats using microdialysis, whereas Badanich et al. (2006) found that P35 rats had lower and P45 rats had higher baseline dopamine levels in the nucleus accumbens compared to adults. However, group differences in baseline TH levels have been implicated in differential sensitivity to amphetamine, such that rats that are less sensitive to novelty also exhibit reduced locomotor activity after amphetamine treatment (e.g., Verheij & Cools, 2008). Reduced TH-ir (Verheij & Cools, 2008) and intracellular dopamine (Verheij et al., 2008) in this region has been found in rats selected for reduced sensitivity to novelty and amphetamine. Thus, reduced dopamine in the nucleus accumbens core may have a role in hypoactivity after acute amphetamine treatment in adolescence.

In the caudate, TH-ir was reduced at P40 and P50, but no difference was found between P30 rats and adults. This is of note, given that greater changes in dopamine receptor density during adolescence have been reported for the caudate than for the nucleus accumbens (Andersen & Teicher, 2000; Teicher, Andersen, & Hostetter, 1995). Moreover, dopamine receptor density in this region was found to peak at P28 to levels higher than for other stages of adolescence and for adulthood (Tarazi & Baldessarini, 2000). Potential implications of age differences in TH-ir in this region are less clear than differences in the nucleus accumbens, as locomotor activating effects of amphetamine are more strongly associated with dopamine release in the nucleus accumbens, whereas stereotypy induced by higher doses of amphetamine is more strongly associated with activity in the caudate (Kelly, Seviour, & Iversen, 1975; Rebec et al., 1997). However, decreased TH-ir observed at 40 and 50 days of age is consistent with evidence that adolescent rats (P43) have reduced basal and amphetamine-induced dopamine release in the dorsal striatum compared to adults (Laviola et al., 2001).

TH-ir was also reduced in both regions of the medial prefrontal cortex (mPFC) in adolescence compared to adulthood. These results are consistent with the suggestion that dopamine synthesis in the mPFC in adolescence may be reduced to compensate for increasing dopamine inputs to this region in adolescence (reviewed in Spear, 2000). An adolescent decline in TH-ir in the mPFC may be involved in altered sensitivity to amphetamine in adolescence, as dopamine release in this region is inversely related to dopamine release in the nucleus accumbens (Pierce & Kalivas, 1997). Because TH-ir in the present study represents basal expression, it is not clear how dopamine release in either region would be influenced by amphetamine treatment. However, a recent study

showed that activation of dopamine D2 receptors had opposite effects on synaptic responses of accumbal medium spiny neurons after PFC stimulation in slices from adolescent and adult rats (Benoit-Marand & O'Donnell, 2008), suggesting that age-specific changes in dopamine synthesis may be involved in hyporesponsiveness to amphetamine.

Conclusions

Data from the present set of experiments support previous findings that adolescence is a period of altered sensitivity to psychostimulants. We demonstrated that adolescent rats were less active than adults after acute amphetamine and that this age difference was attenuated with a higher dose or with repeated amphetamine treatment. Thus, adolescents appear to have a higher threshold for behavioral activation after amphetamine than adults, which is consistent with reduced TH-ir in prefrontal and striatal brain regions during adolescence. Moreover, hypoactivity in adolescent females was not affected by ovariectomy, suggesting that reduced activity in adolescent females is not caused by differences in circulating gonadal hormones. Thus, further studies of differences in sensitivity after acute drug treatment and rapid drug -induced neural plasticity in adolescence may help explain increased sensitivity to drug abuse during this period of development.

RATIONALE FOR STUDY 2

In study 1, I argued that the locomotor hypoactivity to amphetamine during adolescence may reflect a higher response threshold for adolescent than for adult rats, given that hypoactivity was reduced with a second injection of 0.5 mg/kg of amphetamine and eliminated with administration of a higher (1.5 mg/kg) dose of amphetamine. The threshold and the intensity of psychostimulant responses are determined by the mesocorticolimbic dopamine system (Pierce & Kalivas, 1997) and results of study 1 found that adolescent rats had less tyrosine hydroxylase than adults in key components of this system, including the nucleus accumbens and the medial prefrontal cortex. The nucleus accumbens is the principal site for initiating the locomotor activating effects of amphetamine (e.g., Sellings & Clarke, 2003), which suggests that the ongoing development of this region may account for age differences in the locomotor activating effects of the first and the second injection of amphetamine in study 1.

Study 2 was designed to investigate this possibility by addressing two hypotheses. First, if age differences in locomotor activating effects of amphetamine reflect developmental differences in the nucleus accumbens, then age differences in locomotor activity will be observed after injections of amphetamine directly into this region. Second, if increased activity to a second injection of systemic amphetamine in study 1 is initiated in the nucleus accumbens, then an increase in activity from the first to the second injection of amphetamine into the nucleus accumbens will be observed only in adolescent rats. Only male rats were included in study 2 because of our finding that the pattern of age differences was similar for males and females in study 1, in that both sexes were hypoactive only to the lower dose of amphetamine compared to adults.

CHAPTER 3: HEIGHTENED LOCOMOTOR-ACTIVATING EFFECTS OF AMPHETAMINE ADMINISTERED INTO THE NUCLEUS ACCUMBENS IN ADOLESCENT RATS

Abstract

Enhanced risk for drug abuse in adolescence has been associated with developmental changes in the nucleus accumbens (NAc), which is associated with addictive and locomotor activating effects of psychostimulants, but studies of targeted drug injection into the developing NAc are lacking. Rats were given injections of amphetamine (0, 3, or 6 μg/side) directly into the NAc in early (postnatal day 30; P30) or late (P45) adolescence, or in adulthood (P75) and locomotor activity was recorded during two 1 h sessions, 48 h apart. Amphetamine increased locomotor activity at all ages. P30 rats were more active than adults only at the 6 μg/side dose, whereas P45 were more active than adults only at the 3 μg/side dose, indicating that locomotor response magnitude is highest on P30 and sensitivity to low amphetamine doses is highest on P45. Heightened sensitivity of the NAc to amphetamine is consistent with increased risk for addiction during adolescence.

Introduction

In people, risk for drug abuse and addiction is higher during adolescence compared to any other age group, with adolescents exhibiting higher rates of drug abuse and faster transition from drug use to addiction compared to adults (reviewed Spear, 2000; Wu & Schlenger, 2003a). Increased risk for drug abuse has been associated with greater sensitivity to rewarding and reduced sensitivity to aversive effects of psychostimulants in adolescents than in adults (Ernst & Fudge, 2009; Ernst et al., 2009). Differences in psychostimulant sensitivity also are found in adolescent rodents, which exhibit higher sensitivity to rewarding and reduced sensitivity to aversive effects of drugs compared to adults (Badanich et al., 2006; Brenhouse, Sonnteg, & Andersen, 2008; Infurna & Spear, 1979; Schramm-Sapyta et al., 2006), suggesting that rodent models of adolescence are well suited for investigation of age differences in risk for drug abuse and addiction.

Locomotor activity is a commonly used measure for assessing psychostimulant sensitivity, however there is discrepancy across studies regarding age differences in the locomotor-activating effects of psychostimulants. Whereas higher activity levels are found in adolescents than in adults with drugs that block dopamine uptake (e.g., cocaine, methylphenidate, and nomifensine), hypoactivity is found in adolescents with drugs that enhance locomotor activity by increasing dopamine release (e.g., amphetamine, MDMA) (Badanich et al., 2006; Bolanos et al., 1998; Mathews, Waters, & McCormick, 2009; Walker et al., 2010). The pharmacological basis for these effects is not well-understood, although it has been proposed that pharmacokinetic factors, that is, variation across drugs in reaching neural targets, may be an important source of age-specific effects.

Pharmacokinetic changes may account for dosage effects, as studies using amphetamine have found adolescents to be less active than adults at low drug doses and as active as adults at higher doses (Adriani & Laviola, 2000; Bolanos et al., 1998; Mathews & McCormick, 2007; Mathews et al., 2009). Nevertheless, studies that have examined both behavioural and pharmacokinetic effects of psychostimulants have not found a relationship between brain levels of drug and locomotor activity that could explain age differences (Frantz et al., 2006; Spear & Brake, 1983; Zombeck et al., 2009). Further, pharmacokinetic factors do not readily explain the effects of repeated amphetamine treatment, whereby adolescents, and not adults, showed a significant increase in locomotor activity to a second dose of amphetamine (Mathews et al., 2009). Further, despite initial hyporesponsiveness, adolescents showed evidence of long-lasting sensitization to amphetamine that was not found when the same treatment regimen was administered in adulthood (Mathews, Kelly, & McCormick, 2010).

It is likely that age differences in the central nervous system underlie developmental differences in drug-related behaviour. Locomotor activating effects of amphetamine are associated with amphetamine's actions in the mesocorticolimbic dopamine system, which undergoes extensive remodelling in adolescence (reviewed in Andersen, 2005; Chambers et al., 2003; Crews, He, & Hodge, 2007). The mesolimbic dopamine system is comprised of dopaminergic projections from the ventral tegmental area to the nucleus accumbens (NAc) and the medial prefrontal cortex (mPFC), as well as the amygdala and the hippocampus (Fallon, 1988; Lewis & O'Donnell, 2000; Swanson, 1982). Amphetamine results in prolonged actions of dopamine at post-synaptic targets by increasing dopamine release through actions at the dopamine transporter (reviewed in

Fleckenstein, Volz, Riddle, Gibb, & Hanson, 2007). The NAc is a key structure for amphetamine's locomotor activating effects. Lesions of dopamine terminals in the core of the NAc block locomotor activating effects of amphetamine (Sellings & Clarke, 2003) and injections of amphetamine directly into the NAc are sufficient to produce a robust increase in locomotor activity (e.g., Cador, Bjijou, & Stinus, 1995; Dougerty & Ellinwood, 1981; Vezina & Stewart, 1990). The NAc undergoes changes in dopamine receptor and transporter density, dopamine autoreceptor and dopamine transporter sensitivity, changes in basal dopamine and cAMP levels, as well as in dopamine receptor coupling to cAMP in adolescence (Andersen, 2002; Andersen, Rutstein, Benzo, Hostetter, & Teicher, 1997; Badanich et al., 2006; Stanwood, McElligot, Lu, & McGonigle, 1997; Tarazi & Baldessarini, 2000b; Tarazi, Tomasini, & Baldessarini, 1998; Walker et al., 2010). Nevertheless, it is not clear how these changes relate to age differences in sensitivity to amphetamine.

In adults, much of what is known about effects of psychostimulants in different components of mesocorticolimbic circuitry has been mapped using targeted drug injection into specific neural regions. Studies of targeted drug injection in adolescents are lacking. The one study that has used intra-cerebral drug injection for investigation of age differences found greater locomotor activity in early adolescent than adult rats to an NMDA receptor antagonist delivered directly into the NAc (Frantz & Van Hartesveldt, 1999b), which suggests that there are functional differences in the NAc that can be attributed to developmental differences between adolescents and adults. It is important to investigate the developmental differences in the responsiveness of the NAc to drugs of abuse, considering its critical role in the development of drug addiction (Nestler, 2005).

In the present study we investigated whether age differences in the locomotor activating effects of amphetamine would be found when delivered directly into the NAc. If age differences are observed under these conditions, they likely reflect the maturational status of the NAc, and counter the possibility that age differences are simply a reflection of the systemic route of administration.

Method

Animals

Long Evans male rats (n = 72) were purchased from Charles River Laboratories (St. Constant, QC, Canada) and arrived at our facility on postnatal day 22 (P22), P35, or P65. These ages were selected so that by time of testing, ages would represent early adolescence (pre-pubertal, P30), late adolescence (post-pubertal, P45), and adulthood (P75) based on the classifications of Tirelli and colleagues (Tirelli et al., 2003). Rats were housed two per cage until surgery at which point they were singly housed with food and water available *ad libitum* and on a 12 h (lights on at 8 am) light cycle. All experiments were in compliance with National Institutes of Health (NIH) and the Canadian Council of Animal Care (CCAC) guidelines, and were approved by the Brock University Animal Care and Use Committee.

Surgery

Rats underwent stereotaxic surgery for bilateral cannulae implantation into the core of the nucleus accumbens on P25, P40, or P70. Rats were anaesthetized with a ketamine/xylazine mixture and guide cannulae were implanted into the nucleus accumbens using age-appropriate coordinates (P70: AP 10.6 from lambda, ML 1.5, and DV – 5.8; P25: AP 9.3, ML 1.5, DV, 4.0; P40: AP 9.6, ML 1.5, DV 5.2). Coordinates for adult rats were based on the atlas of Paxinos and Watson (Paxinos & Watson, 2005) and

coordinates for the adolescent groups were determined from the initial pilot surgeries. Guide cannulae, 16 mm in length, were constructed from 23 gauge needles and positioned 1 mm above the injection site, such that the injection needle (30 gauge, 17 mm in length) protruded 1 mm below the tip of the guide cannulae. Guide cannulae were secured in place using stainless-steel jewellery screws and dental acrylic and were plugged with removable pins. Rats were given 5 days to recover from surgery before the start of testing.

Locomotor activity

Locomotor testing was conducted in four white open top square melamine arenas (58 cm) under indirect red light illumination to reduce anxiety associated with bright lighting. Rats were habituated to the test arena at either P30, P45, or P75, when all rats were given a bilateral injection of saline (0.5 µl/side) into the NAc. Injections were administered over 1 min with Hamilton constant rate micro syringes (CR 700) that were connected to stainless-steel injecting needles by polyethylene PE-10 tubing. The injection needles were removed after an additional minute to allow for diffusion of vehicle into the brain and then rats were placed into the locomotor test arena for 1 h of habituation. Testing began 24 h later (test 1) when rats were either P31, P46, or P76. Rats were given an injection of either saline (0.5 µl/side) or one of two doses of amphetamine (3 or 6 µg in 0.5 µl of saline) into each hemisphere before placement into the test arena. Locomotor activity was recorded using a Sony video camera mounted from the ceiling and connected to a computer tracking system (Smart; Panlab, Spain) that recorded distance traveled in cm. The same procedure was repeated 48 h later (test 2), with rats given the same drug and dose as on test 1. After testing was completed, rats were given a bilateral injection of

0.5 µl of 2.5% methylene blue dissolved in saline directly into the NAc to confirm cannulae placements. Brains were sliced into 50 µm sections, mounted onto slides, and examined under a light microscope for localization of cannulae placements. Only rats with both injection sites terminating into the NAc were included in the analyses, except for one saline-treated rat at P30 for which one cannula was just above the NAc in the caudate nucleus. Injection sites ranged from AP: 11.3 - 10.3; ML: 1.0 - 2.8; DV: 6.0 – 8.0.

Statistics

Analyses were conducted using mixed factor ANOVA (SPSS Inc., Chicago, IL), with the between group factors of Age (P30, P45, or P75) and Drug (saline, 3 or 6 µg/side of amphetamine) and the within group factor of Test Day (test 1, test 2). The sample size for statistical analysis was n = 56. Twelve rats were removed from the analyses because one (or both) cannula was outside the NAc core. Another four rats were removed from analyses because of technical problems associated with injections.

Results

Locomotor activity: Total distance traveled

An Age (P30, P45, P75) X Drug (0, 3, or 6 µg/side of amphetamine) X Test Day (Test 1, Test 2) ANOVA revealed significant Test Day X Drug ($F(2,47) = 5.05$, $p = 0.01$) and Age X Drug ($F(4,47) = 3.98$, $p = 0.007$) interactions, and an Age X Test Day interaction that approached significance ($F(2,47) = 2.95$, $p = 0.06$). Post hoc tests found that activity decreased from test 1 to test 2 only for rats given 3 µg/side of amphetamine ($p = 0.02$), although this difference was not significant when each age was analyzed

separately. For rats given 6 μg/side of amphetamine, the decline in activity from test 1 to test 2 was significant only for P75 rats ($p = 0.003$) (see Figure 3.1 a-c).

Analysis of the Age X Drug interaction revealed a significant effect of dose in P30 rats ($F(2,13) = 22.12$, $p < 0.0001$), with rats given 6 μg/side of amphetamine traveling greater distances than those given either saline ($p < 0.0001$) or 3 μg/side of amphetamine ($p = 0.003$). Rats given 3 μg/side traveled greater distances than those given saline ($p = 0.01$). The effect of dose was also significant on P45 ($F(2,13) = 4.46$, $p = 0.03$), with rats given 3 ($p = 0.01$) or 6 ($p = 0.03$) μg/side of amphetamine traveling greater distances than those given saline. Rats in the 3 μg/side group did not differ from those in the 6 μg/side group in P45 rats. The effect of dose was significant for P75 rats ($F(2,21) = 32.30$, $p < 0.0001$), with rats given 3 ($p = 0.02$) or 6 ($p < 0.0001$) μg/side of amphetamine traveling greater distances than those given saline, and rats given 6 μg/side of amphetamine traveling greater distances than rats given 3 μg/side of amphetamine ($p = 0.001$) (see Figure 3.1 a-c).

Age differences were significant for each drug group. For rats given saline ($F(2,15) = 3.99$, $p = 0.04$), P45 rats traveled greater distances than did P30 rats ($p = 0.01$). There was also a trend for P45 rats to travel more than P75 rats ($p = 0.06$). For rats given 3 μg/side of amphetamine ($F(2,14) = 3.93$, $p = 0.04$), P45 rats traveled greater distances than P75 rats ($p = 0.02$), whereas P30 and P75 rats did not differ. For rats given 6 μg/side of amphetamine ($F(2,18) = 7.45$, $p = 0.004$), P30 rats traveled greater distances than P75 rats ($p = 0.001$), and P45 and P75 rats did not differ (see Figure 3.1 a-c).

Locomotor activity: Percent change from saline

To control for potential effects of baseline differences in locomotor activity, the analyses were repeated for the amphetamine groups' activity expressed as the percent change from that of saline groups. There was a Day X Dose interaction ($F(1, 32) = 7.61$, $p = 0.01$), however, post hoc analysis of the percent increase in activity from saline from test 1 to test 2 was not significant for either dose of amphetamine.

The Age X Dose interaction was significant ($F2,32 = 4.27$, $p = 0.02$): Rats traveled more in the 6 μg/side than the 3 μg/side groups for P30 rats ($t(9) = 3.05$, $p = 0.01$) and for P75 rats ($t(13) = 3.44$, $p = 0.004$), but not for P45 rats. The effect of Age did not reach significance for rats given 3 μg/side of amphetamine ($F(2,14) = 2.19$, $p = 0.15$) (see Figure 3.1 d). For rats given 6 μg/side of amphetamine, the effect of Age was significant ($F(2,18) = 16.41$, $p < 0.0001$), with P30 rats traveling greater distances than P45 ($p < 0.0001$) and P75 ($p < 0.0001$) rats (see Figure 3.1, e).

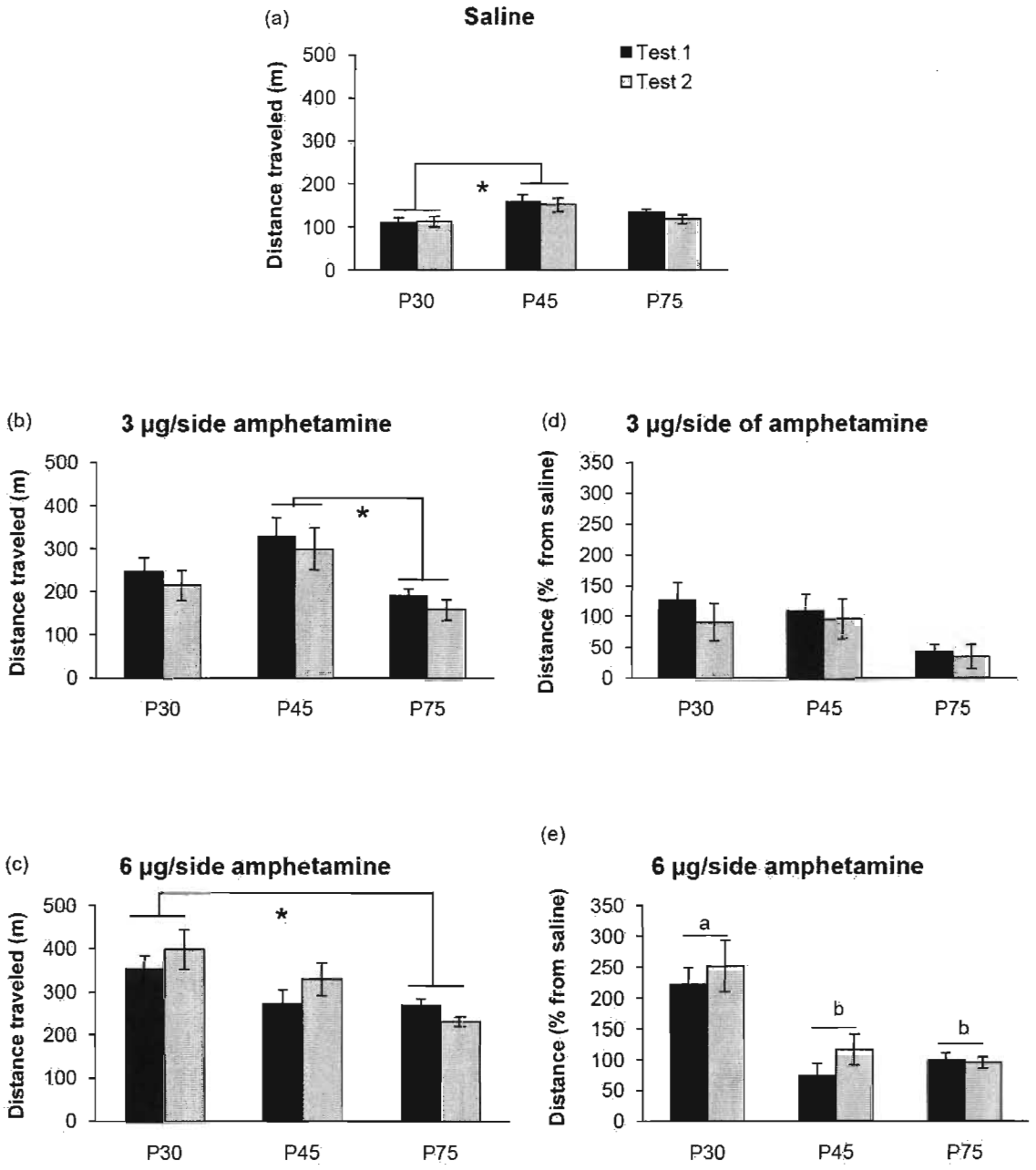

*Figure 3.1. Mean (±SEM) locomotor activity in rats that were given (a) saline, (b) 3 µg/side of amphetamine, or (c) 6 µg/side of amphetamine into the nucleus accumbens. Locomotor activity expressed as the percent change from saline for rats given (d) 3 µg/side and (e) 6 µg/side of amphetamine is also shown. *p < 0.05. Different letters denote a significant difference, p < 0.05.*

Comparison of locomotor activity after systemic vs. intra-accumbens amphetamine

To gauge how intra-NAc dosages compare with systemic administration, we compared locomotor activity in the present study to previous findings (Mathews et al., 2009). For P30 rats, distance traveled after 3 μg/NAc of amphetamine was not different from that after 0.5 mg/kg i.p., although the shorter distance traveled after 0.5 mg/kg i.p. than after 3 μg/NAc on test 1 approached significance ($p = 0.07$). Distance traveled after 6 μg/NAc was not different from that after 1.5 mg/kg i.p. on either test day. For P45 rats, distance traveled after 3 or 6 μg/NAc was higher than after 0.5 mg/kg i.p on both test days (both $p < 0.05$) and was not different from that after 1.5 mg/kg i.p. on either test day. For P75 rats, distance traveled after 6 μg/NAc was not different from that after 0.5 mg/kg i.p. and was less than that after 1.5 mg/kg i.p. on test 1 ($p = 0.06$) and on test 2 ($p = 0.02$). In summary, for P30 rats, 3 μg/NAc = 0.5 mg/kg i.p < 6 μg/NAc = 1.5 mg/kg i.p.; for P45 rats, 0.5 mg/kg i.p. < 3 μg/NAc = 6 μg/NAc = 1.5 mg / kg; for P75 rats, 3 μg/NAc < 0.5 mg/kg i.p = 6 μg/NAc < 1.5 mg/kg i.p (see Figure 3.2).

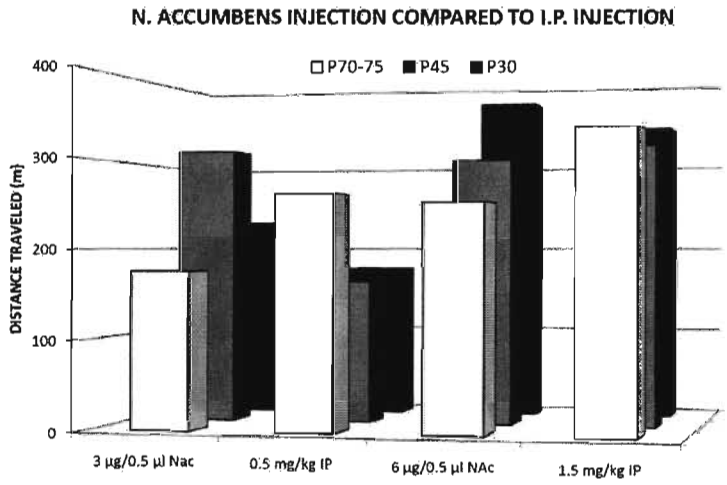

Figure 3.2. Mean locomotor activity for rats given 3 or 6 μg/side of amphetamine (P30, P45, P75) into the nucleus accumbens compared with locomotor activity for rats given 0.5 or 1.5 mg/kg of amphetamine, i.p. (P30, P45, P70) collapsed over two test days.

Discussion

We used targeted drug delivery into the brain to investigate the role of the nucleus accumbens (NAc) in developmental differences in the locomotor activating effects of amphetamine. Compared to saline injection, both doses of amphetamine increased locomotor activity in all age groups on both test days, as reported previously for adult rats (e.g., Cador et al., 1995; Dougerty & Ellinwood, 1981; Vezina & Stewart, 1990). The injection of a higher dose of amphetamine (6 μg/side) led to increased locomotor activity compared to a lower dose (3 μg/side) in both P30 (pre-pubertal, early adolescence) and P75 (adult) rats, whereas activity did not differ between the two doses at P45 (mid-adolescence, post-pubertal). Injections of amphetamine into the NAc have been shown to increase locomotor activity dose-dependently within this dose range in adults (e.g., Cador et al., 1995).

Overall, injection of amphetamine directly into the NAc produced greater locomotor activity in adolescent than in adult rats. The higher locomotor activity of P30 rats compared to P75 rats was significant only with the higher dose, and P30 rats were also more active than P45 rats with the higher dose when activity was expressed as the percent change from saline. In contrast, P45 rats were more active than adults with the lower dose of amphetamine only, and the difference was attenuated when activity was expressed as the percent change from saline. These data suggest that the nature of the age difference in the NAc changes over stages of adolescence, with the magnitude of the locomotor response declining after early adolescence, and sensitivity to lower levels of stimulation increasing in late adolescence before declining to adult levels.

Changes in the locomotor activating effects of amphetamine at different stages of adolescence and in adulthood likely reflect developmental changes in the NAc. In the NAc, there is a continued rise in dopamine transporter (Tarazi et al., 1998), and dopamine D3 receptor (Stanwood et al., 1997) density throughout adolescence, and an overproduction of dopamine D1 and D2 receptors that peaks at P40 before declining to adult levels (Andersen et al., 2000; Tarazi & Baldessarini, 2000b). In addition, basal dopamine (Badanich et al., 2006) and cAMP (Andersen, 2002) levels are elevated in adolescence, whereas dopamine receptor regulation of cAMP is reduced (Andersen, 2002) and dopamine autoreceptors (Andersen et al., 1997) and dopamine transporters (Badanich et al., 2006) function is enhanced in the NAc of adolescent compared to adult rats. Results of the present study suggest that the net effect of these developmental changes is to raise sensitivity of this region to amphetamine in adolescence compared to adulthood. Moreover, age differences in activity cannot be explained by greater diffusion of amphetamine in the brains of adolescent than adult rats because P30 rats, which have smallest brains, were not more active than adults at the low dose of amphetamine, and each age comparison was significant only at one of the two doses.

The results suggest that there is a rightward shift in the dose-response curve of amphetamine in the NAc of adult compared to adolescent rats. We compared locomotor activity after intra-accumbal amphetamine to that found after systemic amphetamine in a previous study (Mathews et al., 2009): Systemic administration of 1.5 mg/kg amphetamine resulted in similar locomotor activity as reported here for 6 μg/side of intra-accumbal amphetamine in P30 rats and higher activity than that for 6 μg/side in adult rats, which indicates that activity after the 6 μg/side dose is not the ceiling for locomotor

activity in adults. Thus, either adults would show increased locomotor activity to an intra-accumbal dose higher than 6 μg/side or the higher activity after 1.5 mg/kg amphetamine than after 6 μg/side of intra-accumbens amphetamine involves actions at other neural sites.

The finding of greater sensitivity of adolescents than adults to intra-accumbal amphetamine is in contrast to the reports of hyposensitivity in adolescence to systemic doses of amphetamine (Adriani & Laviola, 2000; Bolanos et al., 1998; Frantz et al., 2006; Mathews & McCormick, 2007; Mathews et al., 2009; Zombeck et al., 2009). Others have also found that the pattern of age differences in drug-induced behaviour depends on route of administration: Adolescents had greater activity than did adults after intra-NAc injection of the NMDA receptor antagonist MK-801, whereas adults had greater activity than adolescents after systemic injection of MK-801 (Frantz & Van Hartesveldt, 1999a, 1999b). In conjunction with our study, these data suggest that developmental differences in regulatory regions outside of the NAc contribute to locomotor hypoactivity in adolescent rats and to enhanced locomotor activity in adult rats after systemic drug administration. We previously found that adolescents, and not adults, had a significant increase in activity to a second administration of systemic amphetamine (Mathews et al., 2009). Increased locomotor activity to a second dose of amphetamine was not found here using intra-accumbal administration, which also suggests that any heightened plasticity in adolescents to repeated administration involves sites other than the NAc. For example, hippocampal lesions were found to increase locomotor activating effects of amphetamine in adolescent rats (Lanier & Isaacson, 1977) and decrease locomotor activating effects of amphetamine in adult rats (White, Whitaker, & White, 2006). Developmental differences

in the medial prefrontal cortex have also been hypothesized to contribute to altered psychostimulant sensitivity in adolescence (Ernst et al., 2009; Spear, 2000), but this hypothesis has not been investigated directly.

Conclusions

Results of the present study demonstrate that sensitivity of the NAc to amphetamine changes over adolescence, with early adolescent rats (P30) showing the greatest locomotor-activating effects of intra-accumbal amphetamine and late adolescent rats (P45) exhibiting greater locomotor-activating effects compared to adults to the lower dose of amphetamine. Higher sensitivity of the NAc to amphetamine in adolescence than in adulthood has important implications for increased risk for drug addiction during this period, as actions of psychostimulants in the NAc are implicated strongly in addictive effects of drugs. Moreover, our findings provide support for theories of the neural basis of motivated behaviour in adolescence (Chambers et al., 2003; Ernst et al., 2009), which hypothesize that increased sensitivity to reward during adolescence is associated with increased sensitivity of the nucleus accumbens to rewarding effects of psychostimulants during this period of development.

RATIONALE FOR STUDY 3

In study 2, adolescent rats were more active than adults when amphetamine was injected directly into the nucleus accumbens. These results, however, contrast the findings of study 1, in which adolescent rats were less active than adults after a systemic injection of 0.5, and same as adults after 1.5, mg/kg of amphetamine. Thus, either low doses of amphetamine administered peripherally may be less effective at activating the nucleus accumbens in adolescents, or age differences in the locomotor activating effects of systemic amphetamine involve brain regions other than the nucleus accumbens. One possible neural candidate is the medial prefrontal cortex, given my finding of reduced tyrosine hydroxylase expression in the medial prefrontal cortex in adolescent compared to adult rats. Thus, the first aim of study 3 was to investigate age differences in amphetamine-induced activation of the nucleus accumbens and the medial prefrontal cortex in adolescent and adult rats using phosphorylation of CREB and the expression of the immediate early gene c-fos as markers of neural activation.

The second aim of study 3 was to directly test the hypothesis that the medial prefrontal cortex regulates amphetamine-induced locomotor activity in an age-specific way. Dopamine receptor agonists and antagonists were injected directly into this region and the locomotor activating effects of systemic amphetamine were compared in adolescent and adult rats.

CHAPTER 4: ROLE OF MEDIAL PREFRONTAL CORTEX DOPAMINE IN AGE DIFFERENCES IN RESPONSE TO AMPHETAMINE IN RATS: CREB AND FOS EXPRESSION, AND LOCOMOTOR ACTIVITY AFTER INTRA-MPFC INJECTIONS OF DOPAMINERGIC LIGANDS

Abstract

Here we investigate the neural basis for locomotor hypoactivity to amphetamine in adolescent compared to adult rats. Rats aged postnatal day 30 (P30), P45 and P75 were given amphetamine (0.0, 0.5, or 1.5 mg/kg, i.p.) and phosphorylation of CREB (pCREB; experiment 1) and expression of Fos (experiment 2) were measured. In the nucleus accumbens, pCREB and Fos-ir were higher in adolescent than in adult rats, but amphetamine and saline treated rats did not differ. In the prelimbic medial prefrontal cortex (mPFC), the 1.5 mg/kg dose of amphetamine induced more Fos-ir than the 0.5 mg/kg dose and saline only in P30 rats. To further investigate the role of this region in age differences, in experiment 3 the D1 antagonist SCH 23390 or the D2 antagonist raclopride were injected directly into the prelimbic mPFC before a systemic injection of 1.5 mg/kg of amphetamine. Intra-mPFC D2 antagonist did not differ from intra-mPFC saline groups in amphetamine-induced activity, whereas injection of a D1 receptor antagonist reduced amphetamine activity more in P30 than in P45 and P75 rats. In experiment 4, using a lower dose of amphetamine (0.5 mg/kg), an intra-mPFC injection of a D1 receptor agonist increased locomotor activity in adolescent and decreased activity in adult rats. These results suggest that insufficient activation of mPFC D1 receptors may underlie hypoactivity to a low dose of amphetamine and that greater recruitment of D1 receptors is required to achieve adult-like locomotor activity at a higher dose.

Introduction

Most drug use is initiated in adolescence (reviewed in Chambers et al., 2003; Spear, 2000), when progression from recreational use to dependence occurs more rapidly than in adulthood (Clark et al., 1998). Initiation of drug use in adolescence is associated with more severe dependence in later life than is initiation of drug use in adulthood (e.g., Clark et al., 1998; Estroff, Schwartz, & Hoffmann, 1989; Merline et al., 2004; Windle et al., 2008), especially in cases where drug use begins in early compared to later stages of adolescence (Hawkins et al., 1997; Yamaguchi & Kandel, 1984). Increased risk for drug dependence during adolescence may be attributed in part to age differences in drug sensitivity. For example, adolescents report feeling only negligible effects of cocaine at first use, which may lead to higher levels of drug intake during adolescence (Laviola et al., 1999; Weiss et al., 1994). The age differences in sensitivity to psychostimulant effects have been attributed to ongoing development of neural substrates that underlie drug responding (Chambers et al., 2003; Ernst et al., 2009; Spear, 2000).

Rodent models of adolescence have made important contributions to our understanding of age differences in behavioural responses to psychostimulants and of the developmental changes in neural substrates that underlie those responses. Addictive properties of drugs are associated with their actions in the nucleus accumbens (NAc) (Nestler, 2005), a region that is critical for generating reinforcing and locomotor activating effects of psychostimulants (Sellings & Clarke, 2003; Wise & Bozarth, 1987). Using locomotor activity as an index of psychostimulant sensitivity, adolescent rodents are typically found to be less sensitive to a first treatment with 0.5 mg/kg of amphetamine compared to adults (Bolanos et al., 1998; Mathews & McCormick, 2009). Nevertheless,

we found that age differences in activity were eliminated with administration of a higher dose (1.5 mg/kg) of amphetamine (Mathews & McCormick, 2009), indicating that adolescents require more amphetamine to attain adult-like responding. In contrast, when amphetamine was administered directly into the NAc, adolescent rats were found to be more active than adults (Mathews & McCormick, 2009). These findings suggest that the reduced activity in adolescent rats in response to a systemic injection of amphetamine, which results in dispersion of the drug throughout the brain, may involve actions of amphetamine on regions outside of the NAc that modulate the locomotor activating effects of amphetamine. Indeed, the hippocampus was proposed to have an inhibitory effect on amphetamine-induced locomotor activity in adolescence based on the finding that the locomotor activating effects of systemic amphetamine in adolescent rats was enhanced when the hippocampus was lesioned (Lanier & Isaacson, 1977).

Effects of regulatory regions other than the NAc and hippocampus on locomotor activating effects of amphetamine during adolescence have not been investigated. Nonetheless, it has been proposed that ongoing development of the medial prefrontal cortex (mPFC) may be involved in the differential regulation of psychostimulant responding in adolescence compared to adulthood (Ernst et al., 2009; Spear, 2000). The mPFC is a heterogeneous region comprised of cingulate, prelimbic and infralimbic subregions that are highly connected to all components of the mesolimbic dopamine system, including the NAc (Porrino & Lyons, 2000). The mPFC and the NAc are involved in 'approach' toward salient stimuli, which can be manifested as enhanced locomotor activity as well as self-administration of, and increased preference for,

psychostimulants (Alleweireldt, Weber, Kirschner, Bullock, & Neisewander, 2002; Sanchez, Bailie, Wu, Li, & Sorg, 2003; Wise & Bozarth, 1987).

In the mPFC, approach to salient stimuli is associated with D1 dopamine receptors (reviewed in Ernst et al., 2009), which attain peak density in adolescence (Andersen et al., 2000). According to the triadic theory of neurobiology of motivated behaviour in adolescence (Ernst et al., 2009), the modulatory role of the mPFC in adolescence is slanted more strongly toward goal-directed approach behaviours than it is in adulthood, in part because of age differences in D1 dopamine receptors. The distribution of prefrontal D1 receptors also changes in adolescence, with many of the overproduced receptors found specifically on NAc projection neurons in late adolescence and on an unidentified group of cells in early adolescent rats (Brenhouse et al., 2008). In adults, a minimal proportion (< 5%) of D1 receptors are localized to glutamatergic projection neurons to the NAc, with most D1 receptors found on GABAergic interneurons (Brenhouse, Sonntag et al., 2008; Vincent, Khan, & Benes, 1993).

In adults, D1 dopamine receptors in the mPFC are necessary for the expression of locomotor activating effects of amphetamine (Hall et al., 2009), although the potential role of mPFC D1 dopamine receptors for regulating age differences in amphetamine-induced locomotor activity has not been investigated. However, the higher resistance to extinction of cocaine conditioned place preference in adolescent compared to adult rats has been postulated to involve greater effects of D1 dopamine receptors in the mPFC during adolescence (Brenhouse et al., 2010). In addition, intra-mPFC treatment with a D1 receptor agonist in adolescent rats increased conditioned place preference (CPP) for a systemically-administered dose of cocaine that did not produce a preference on its own,

thereby directly implicating mPFC D1 receptors in cocaine's effects in adolescents (Brenhouse et al., 2008). These studies did not involve direct comparisons of D1 receptor agonists and antagonists in adolescent and adult rats however, thus it is not clear to what extent the results were age-specific.

The present experiments were designed to investigate the role of D1 receptor activation in age differences in the actions of amphetamine using several different approaches. In experiment 1, age differences in activation of the NAc and the mPFC were investigated by measuring expression of activated cAMP response element binding (CREB) protein using western blotting. CREB is a constitutively bound transcription factor that regulates amphetamine-induced gene expression when activated by phosphorylation at Ser133 through a D1 receptor dependent mechanism (Konradi, Cole, Heckers, & Hyman, 1994). Based on the finding that adolescent rats are less active than adults after 0.5, but not after 1.5 mg/kg of amphetamine (Mathews & McCormick, 2009), we hypothesized that adolescent rats would have less CREB phosphorylation than adults in the NAc at the 0.5, but not at the 1.5 mg/kg dose of amphetamine. In contrast, we hypothesized that a systemic injection of the 1.5 mg/kg dose of amphetamine would result in more CREB phosphorylation than the 0.5 mg/kg dose in the mPFC in adolescent rats, based on the role of mPFC D1 receptors in dose-dependent cocaine place preference in adolescents (Brenhouse et al., 2008). This experiment also provided the opportunity to investigate age differences in hormonal responses to amphetamine, as amphetamine is known to alter concentrations of testosterone and corticosterone in adulthood (Budziszewska, Jaworska-Feil, & Lason, 1996; Tsai et al., 1996), and concentrations of these hormones in circulation in turn modulate the behavioural effects of amphetamine

(Forgie & Stewart, 1994b; Piazza et al., 1991). Gonadal and adrenal hormonal systems are undergoing maturation in adolescence (reviewed in Kuhn et al., 2010; McCormick et al., 2010), but age differences in the regulation of these hormones by amphetamine are not well understood.

In experiment 2, we used immunohistochemistry to measure expression of the immediate early gene c-fos, which is dependent on amphetamine-induced dopamine release and is regulated by CREB phosphorylation (Konradi et al., 1994; LaHoste, Ruskin, & Marshall, 1996; Robertson, Peterson, Murphy, & Robertson, 1989). We also extended our analysis of neural regions to different subregions of the mPFC, including the Cg1, Cg2 and the prelimbic subregions. In experiments 3 and 4, we directly compared the effects of intra-mPFC injections of a D1 dopamine receptor agonist and antagonist in adolescent and adult rats to determine whether the effects of these drugs on the locomotor activating effects of systemic amphetamine would depend on age. In experiment 3, we tested the hypothesis that D1 receptors are involved in regulating the locomotor activating effects of the 1.5 mg/kg dose of amphetamine to a greater extent in adolescent than in adult rats. In experiment 4, we tested the hypothesis that the reduced locomotor activity at the 0.5 mg/kg dose of amphetamine in adolescent rats is associated with reduced activation of prelimbic D1 receptors and that an intra-mPFC injection of a D1 agonist would increase locomotor activating effects at this dose of amphetamine in adolescent rats compared to in adult rats.

Method

Experiments 1 – 4

Animals

The experiments used 219 male Long-Evans rats purchased from Charles River Laboratories (St. Constant, QC) and arrived at our colony on postnatal day 22 (P22), P37±2, or P67±2. These ages were selected so that by time of testing ages would represent early adolescence (pre-pubertal, P30), late adolescence (post-pubertal, P45), and adulthood (P75) based on the classifications of Tirelli and colleagues (Tirelli et al., 2003). Rats were pair housed in plastic cages (46 cm×24 cm×20 cm) on a standard 12h:12h light-dark cycle, with lights on at 8 a.m. Food and water were freely available in the home cage. All experiments were in compliance with National Institutes of Health (NIH) and the Canadian Council of Animal Care (CCAC) guidelines, and were approved by the Brock University Animal Care and Use Committee.

Test arenas (same for experiments 1-4)

Every effort was made to keep conditions constant across experiments to facilitate the applicability of findings with pCREB and Fos-ir in experiments 1 and 2 for interpretation of age differences in locomotor activity in experiments 3 and 4. Previous studies have found that the pattern of amphetamine-induced immediate early gene expression depends on whether testing occurs in the home cage or in a separate chamber (Badiani et al., 1998). Thus, for all experiments, injections occurred outside the home cage and rats were kept in chambers used for measurement of locomotor activity. The chambers consisted of four white open-top melamine arenas (58 cm X 58 cm X 58 cm) that were illuminated indirectly by red light to attenuate anxiety related to bright lighting,

and with white noise in the background. Chambers were cleaned with 50% ethanol after each session. All experiments were conducted between 0900 and 1700h.

Experiment 1 Method

Drug administration

64 rats were used for experiment 1. On day 1 (P30, P45, or P75), all rats were given an intraperitoneal (i.p.) injection of 0.9% saline before placement into the test arena for 1 h of habituation. The next day (P31, P46, or P76), rats were randomly assigned to groups that had an i.p. injection of either saline, 0.5 (Amph 0.5) or 1.5 (Amph 1.5) mg/kg of amphetamine (n = 6-10 rats/group) before placement into the arena for 30 min. Rats were euthanized by rapid decapitation immediately after removal from the test arena for collection of brain tissue and blood samples (see below).

Western blotting

Brains were rapidly removed and a 1 mm section (~10.40 - 11.40 mm from lambda according to Paxinos & Watson, 2005) was dissected using stainless steel Brain Matrices (coronal with 1 mm spacers; Ted Pella, Inc.). Tissue punches (1.19 mm) were taken from the core and shell of the NAc and from the Cg1 subregion of the mPFC. Samples were homogenized in lysis buffer and centrifuged for 10 min at 16,000g. The pellet was discarded and the supernatant was dissolved in equal volume of Lammeli buffer (buffer solutions adapted from Konradi, 2003), heated for 5 min at 70°C, and centrifuged again for 10 min at 16,000g. Samples were resolved using 12% SDS-PAGE and transferred to PVDF membrane (Millipore). Membranes were reversibly stained using Ponceau-S (Sigma-Aldrich) for determination of total protein for use as a loading control (Aldridge, Podrebarac, Greenough, & Weiler, 2008). The membranes were

blocked in 5% BSA in TBS-T for 1 h at room temperature and probed with pCREB primary antibody (1:1000; Abcam) dissolved in TBS-T overnight at 4°C. Membranes were washed thoroughly with TBS-T and incubated in Alexa Fluor 488 (Invitrogen) secondary antibody (1:5000) for 45 min at room temperature. Membranes were washed and imaged wet using the VersaDoc Imaging System (Bio-Rad). Staining intensity (intensity X mm) of each band was analyzed using the Quantity One imaging program for the Versa Doc (Bio-Rad).

Enzyme-linked immunosorbent assay (ELISA)

Trunk blood was collected in ice-chilled tubes containing EDTA. Samples were centrifuged at 3000 rpm for 10 min and plasma was collected and stored at -20°C for later analysis with ELISA kits. Samples were prepared and analyzed according to kit instructions for corticosterone (Neogen Corporation, #402810) and testosterone (Neogen Corporation, #402510). Briefly, steroid hormones were extracted using N_2, diluted in extraction buffer, and assayed in duplicate using the appropriate kit. Plates were read at 650 nm using a Synergy HT microplate reader (Bio-Tek). Typical intra- and inter-assay CV for these kits is $\leq$ 10%.

Statistics

Analyses of corticosterone and testosterone concentrations were conducted using factorial ANOVA with the between-group factors of Age (P30, P45, P75) and Drug (Saline, Amph 0.5, Amph 1.5). One animal was dropped from the analysis because of a technical error in sample preparation. Analyses for phosphorylated CREB (pCREB) were conducted using factorial ANOVA with the between-group factors of Age (P30, P45, P75) and Group (Saline, Amph 0.5, and Amph 1.5). Only brains with sufficient protein

concentrations were used for analysis, which resulted in 4 to 8 animals in each group. Post-hoc analyses were conducted using F-tests for simple effects and Fisher's protected least squares difference tests.

Experiment 1 Results

Endocrine measures

Testosterone. There was an effect of Age on plasma testosterone concentrations ($F(2,45) = 21.87$, $p < 0.0001$): P75 rats had higher concentrations than P30 and P45 rats (both $p < 0.0001$) and P45 had higher concentrations than P30 ($p = 0.04$) rats (see Figure 4.1 a). There was no effect of drug or interaction of age and drug.

Corticosterone. For plasma corticosterone, there was an effect of Age ($F(2,44) = 7.07$, $p = 0.002$), an effect of Drug ($F(2,44) = 11.85$, $p < 0.0001$), and no interaction. P45 rats had higher corticosterone concentrations than P30 ($p = 0.008$) and P75 ($p = 0.001$) rats. Rats in the Amph 1.5 group had higher corticosterone concentrations than rats in the Saline ($p = 0.001$) and Amph 0.5 ($p < 0.0001$) groups. Rats in the Amph 0.5 group did not differ from rats in the Saline group (see Figure 4.1 b).

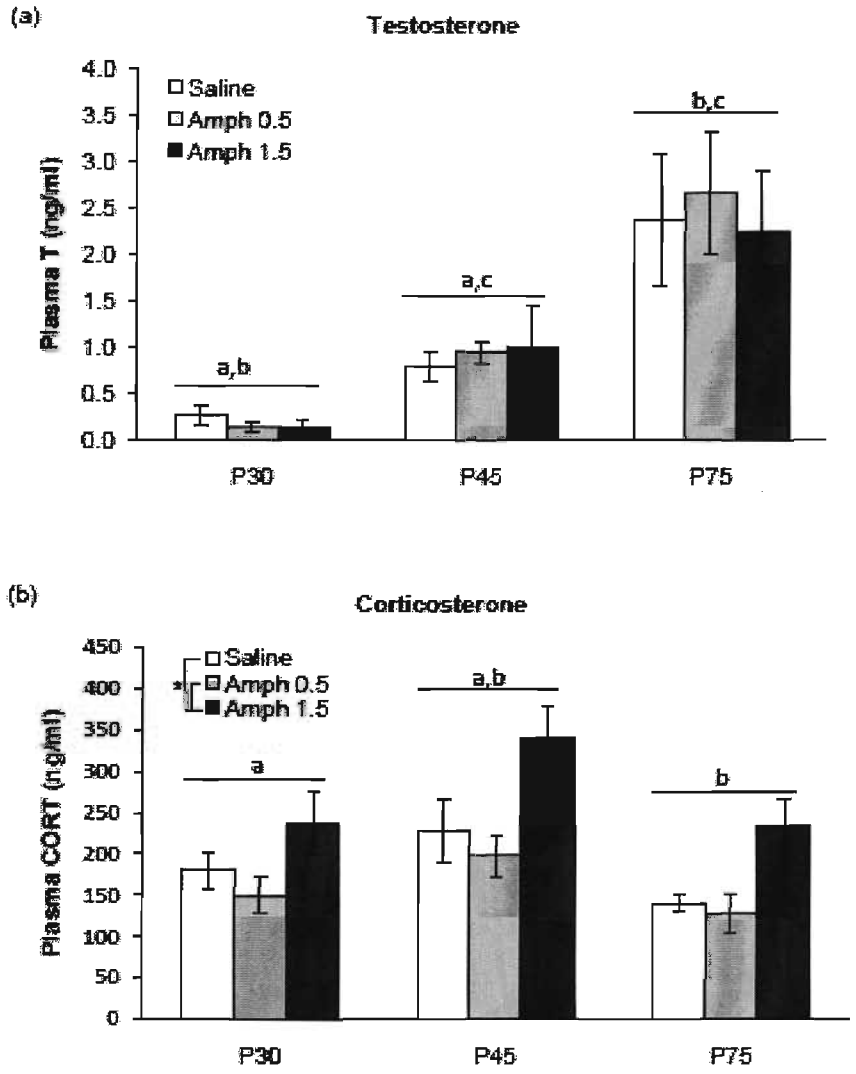

Figure 4.1. Mean (±SEM) plasma (a) testosterone and (b) corticosterone concentrations in P30, P45 and P75 rats 30 min after treatment with saline or amphetamine (0.5 or 1.5 mg/kg). Matched letters indicate a significant group difference.

cAMP response element binding (CREB) protein phosphorylation

Nucleus accumbens. For pCREB in the NAc core, there was an effect of Age only ($F(2,39) = 7.28$, $p = 0.002$): P75 had lower pCREB than P30 ($p = 0.001$) and P45 ($p = 0.005$) rats, and P30 and P45 rats did not differ (see Figure 2 a). In the shell, there an effect of Age ($F(2,40) = 4.48$, $p = 0.02$) only: P45 rats had higher pCREB than P30 rats ($p = 0.008$) and there was a trend for higher pCREB for P45 than for P75 rats ($p = 0.07$) (see Figure 4.2 b).

Medial prefrontal cortex (Cg1). For pCREB in the mPFC, there an effect of Age ($F(2,43) = 5.42$, $p = 0.008$) and an Age X Drug interaction ($F(4,43) = 3.11$, $p = 0.03$). An effect of Age ($F(2,12) = 4.67$, $p = 0.03$) was found for rats in the Saline group: P30 rats had higher pCREB than P45 ($p = 0.05$) and P75 ($p = 0.01$) rats. An effect of Age ($F(2,13) = 4.07$, $p = 0.04$) was found for rats in the Amph 1.5 group: P30 rats had higher pCREB than P75 rats ($p = 0.01$) and there was a trend for higher pCREB in P45 than in P75 rats ($p = 0.07$) rats (see Figure 4.2 c). Age differences were not found for rats in the Amph 0.5 group.

For P30 rats, the effect of Drug ($F(2,17) = 6.67$, $p = 0.007$) was that rats in the Amph 0.5 group had lower pCREB than rats in the Saline ($p = 0.004$) and Amph 1.5 ($p = 0.02$) groups (see Figure 4.2 c). For P45 and P75 rats, the effect of Drug was not significant.

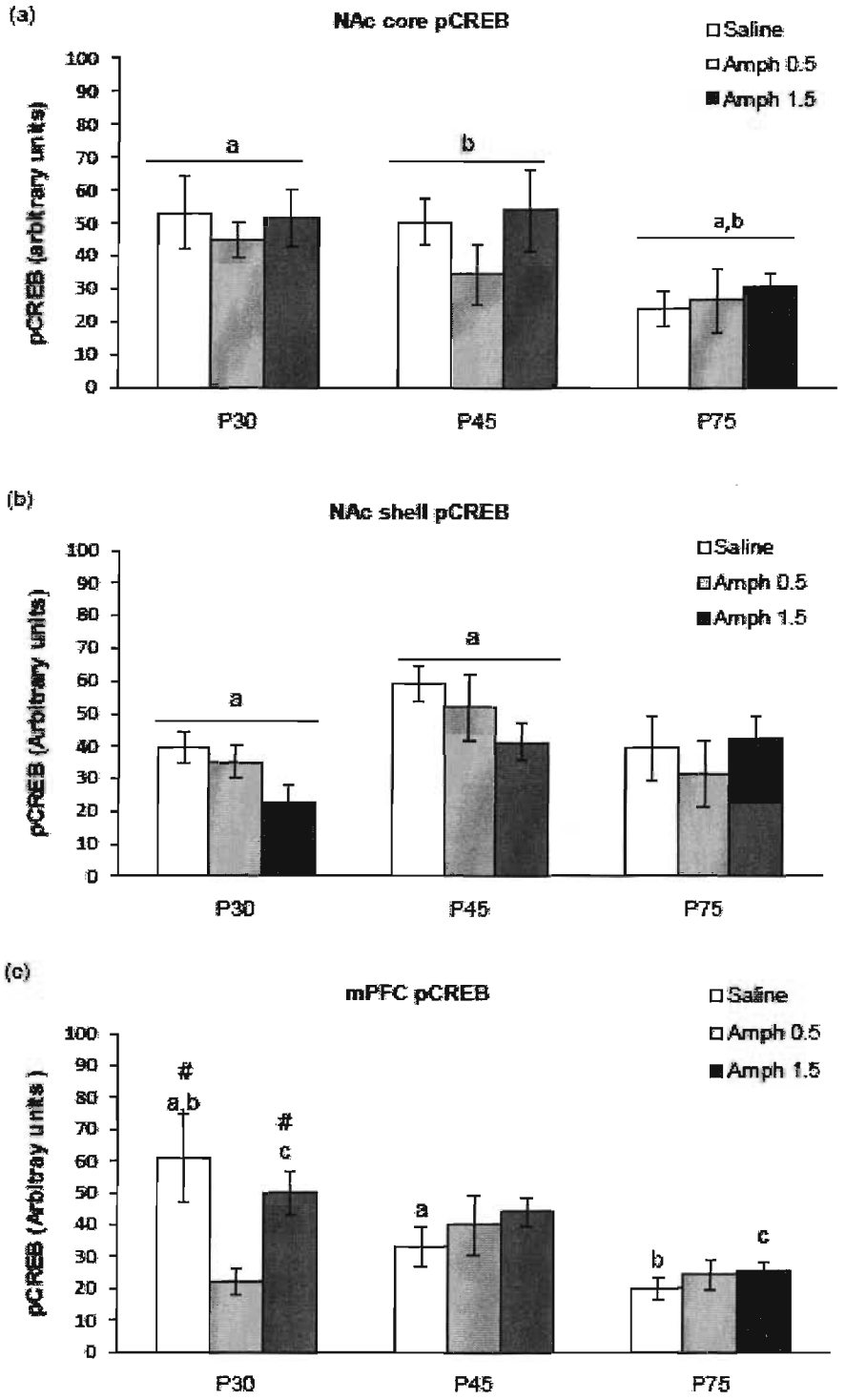

Figure 4.2. Mean (±SEM) staining intensity for pCREB in the (a) nucleus accumbens core, (b) nucleus accumbens shell, and (c) the medial prefrontal cortex; #Higher than 0.5 mg/kg ($p < 0.05$). Same letters indicate a significant group difference.

Experiment 1 Discussion

Overall, adolescent rats were found to have lower basal concentrations of testosterone and higher basal concentrations of corticosterone than adult rats, amphetamine did not have age-specific effects on the release of either hormone, and 1.5 mg/kg amphetamine increased corticosterone concentrations in all age groups. These results are consistent with previous reports of the progressive rise in testosterone through puberty (Korenbrot, Huhtaniemi, & Weiner, 1977) and with findings in adult rats of enhanced corticosterone release only with doses higher than 0.5 mg/kg (Swerdlow, Koob, Cador, Lorang, & Hauger, 1993).

The only effect of drug group on pCREB was in the mPFC in P30 rats, whereby the lower pCREB was found for rats that had 0.5 mg/kg compared to 1.5 mg/kg amphetamine and saline. Amphetamine did not increase pCREB at any dose in the NAc, indicating that CREB phosphorylation is not sensitive enough for detecting drug effects at these doses. Overall pCREB, however, was higher in adolescent than in adult rats in all regions. These findings are consistent with reports of a higher dopaminergic tone in the NAc and the mPFC during adolescence, as evidenced by higher basal levels of cAMP (Andersen, 2002) and greater dopamine receptor density (Andersen et al., 2000) in adolescent compared to adult rats.

In experiment 2 we investigated age differences in amphetamine-induced neural activation of the NAc and the mPFC using Fos, the protein product of the immediate early gene, c-fos. Although Fos expression is strongly associated with phosphorylation of CREB by amphetamine (C. Konradi et al., 1994), Fos-ir is not always correlated with pCREB (e.g., Shiromani, Magner, Winston, & Charness, 1995; Turgeon, Pollack, &

Fink, 1997), and Fos-ir was found to increase in response to acute amphetamine even when pCREB did not change (Turgeon et al., 1997), indicating that c-Fos may allow for improved detection of amphetamine actions in the brain. We also extended the neural regions examined to include the prelimbic subregion as it may be more responsive to psychostimulant treatment than the Cg1 region (Mazei, Pluto, Kirkbride, & Pehek, 2002).

Method Experiment 2

Drug administration

54 rats were used for Experiment 2. On day 1 (P30, P45, or P75), all rats were given an i.p. injection of 0.9% saline before placement into the test arena for 1 h of habituation. The next day (P31, P46, or P76), rats were randomly assigned (n = 6 rats/group) to intraperitonal injection groups of either saline, 0.5 (Amph 0.5) or 1.5 (Amph 1.5) mg/kg of amphetamine before placement into the arena for 1 h.

Immunohistochemistry

After removal from the test arena, rats were deeply anesthetized and transcardially perfused with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were placed in a 30% sucrose and paraformaldehyde solution until they sank. Coronal sections (40 μm thick) were collected throughout the mPFC, including the prelimbic (~11.7 – 12.7 mm from lambda according to Paxinos and Watson (2005) and cingulate cortex area 1 and 2 (Cg1 and Cg2) subregions, and the NAc core and shell (~10.4 – 11.4 mm from lambda according to Paxinos and Watson (2005) using a cryostat (ThermoShandon). Every fourth section was collected for the prelimbic region and every sixth section was collected from the remaining regions for immunohistochemistry, resulting in a total of

four sections within each set of coordinates. The sections were stored in cryoprotectant at -20°C until the immunohistochemistry was performed.

Free floating sections were washed stringently in PBS with 0.2% Triton X (PBSx, pH 7.4), then in 0.3% hydrogen peroxide, and then again in PBSx. Next, the tissues were incubated for 24 hr at 4°C in 1% NGS and Fos primary antibody diluted at 1:2000 (Santa Cruz). After incubation, the sections were washed three times in PBSx before incubation for 75 min in biotinylated anti-rabbit immunoglobulin secondary antibody (Vector Laboratories) diluted at 1:300 (Vector Laboratories). The sections were again washed in PBSx and placed for 30 min in Avidin–Biotin Complex (Vector Laboratories). After another three washes in PBSx, tissues were placed in diaminobenzidine solution according to the instructions on the substrate kit (DAB SK-4100, Vector Laboratories) for 5 min. Immunostained sections were mounted onto slides, dried, and coverslipped with Permount. Sections for the mPFC (prelimbic, Cg1, Cg2) were photographed at 200X magnification and NAc core and shell at 400X magnification with a Nikon Eclipse brightfield microscope and all stained cells were counted. The same size area was counted for all regions and for all ages at 200X (500 μm X 500 μm) and 400X (250 μm X 250 μm) magnification. Measures for each structure were averaged across hemispheres and sections, and rats for which there were fewer than three measures for a structure were excluded, resulting in n = 5±1 for each brain region and for each group.

Statistics

Factorial ANOVAs were conducted for each brain region separately, with the between group factors of Age (P30, P45, P75) and Drug (Saline, Amph 0.5, Amph 1.5).

Post-hoc analyses were conducted using F-tests for simple effects and Fisher's protected least squares difference tests.

Experiment 2 Results

Nucleus accumbens (*NAc*)

Core. For Fos-ir cell counts in the NAc core, there was an effect of Age ($F(2,43) = 3.58$, $p = 0.04$) and an effect of Drug ($F(2,43) = 4.21$, $p = 0.02$). P30 rats had more Fos-ir cells than P75 rats ($p = 0.006$) and there was a trend for more Fos-ir cells in P30 compared to P45 rats ($p = 0.06$) (see Figure 4.3 a). Rats in the Amph 1.5 group had more Fos-ir cells than rats in the Amph 0.5 group ($p = 0.005$) (see Figure 4.3 a).

Shell. For Fos-ir cell counts in the NAc shell, there was a significant Age X Drug interaction ($F(4,42) = 2.58$, $p = 0.05$). Follow-up analyses found an effect of Age ($F(2,15) = 5.12$, $p = 0.02$) only in the Saline group: P45 rats had more Fos-ir cells than P30 ($p = 0.05$) and P75 ($p = 0.007$) rats (see Figure 4.3 b).

For P30 rats, the effect of Drug approached significance ($F(2,13) = 3.59$, $p = 0.057$): rats in the Amph 1.5 group had more Fos-ir cells than rats in the Amph 0.5 group and rats in the Amph 1.5 group had more Fos-ir cells than rats in the Saline group ($p = 0.06$). For P45 and P75 rats, the effect of Drug was not significant.

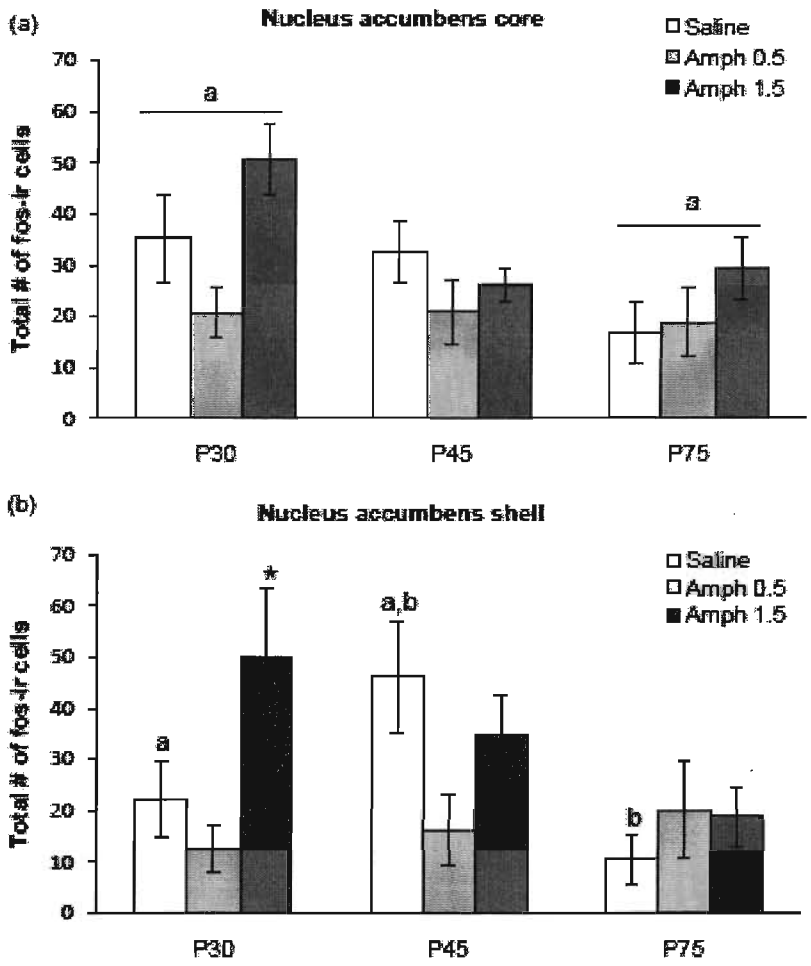

*Figure 4.3. Mean (±SEM) number of Fos-ir cell counts for the nucleus accumbens (a) core and (b) shell; *Higher than Amph 0.5 (p = 0.03). Same letters indicate a significant difference.*

Medial prefrontal cortex (mPFC)

Prelimbic. For Fos-ir cell counts in the prelimbic cortex, there was a main effect of Drug ($F(2,40) = 6.43$, $p = 0.004$) and an Age X Drug interaction ($F(4,40) = 2.78$, $p = 0.04$). Follow-up analyses did not find an effect of Age at any dose. For P30 rats, the effect of Drug ($F(2,12) = 7.00$, $p = 0.01$) was that rats in the Amph 1.5 group had more

Fos-ir cells than rats in the Saline ($p = 0.004$) and Amph 0.5 ($p = 0.01$) groups. For P45 and P75 rats, the effect of Drug was not significant (see Figure 4.4 a).

Cg1. For Fos-ir cell counts in Cg1, there was an effect of Age only ($F(2,40) = 6.45$, $p = 0.004$): P30 rats had more Fos-ir cells than P45 ($p = 0.002$) and P75 ($p = 0.01$) rats (see Figure 4.4 b).

Cg2. For Fos-ir cell counts in Cg2, there was an interaction of Age and Drug ($F(4,40) = 2.95$, $p = 0.03$). An effect of Age ($F(2,13) = 5.27$, $p = 0.02$) was found only for rats in the Amph 0.5 group, with P75 having more Fos-ir cells than P30 ($p = 0.02$) and than P45 ($p = 0.01$) rats (see Figure 4.4 c). For P30 rats, follow-up analyses found that the effect of Age approached significance ($F(2,13) = 3.48$, $p = 0.06$): Rats in the Amph 1.5 group had more Fos-ir cells than rats in the Saline and Amph 0.5 groups. The effect of Drug was not significant for P45 or P75 rats.

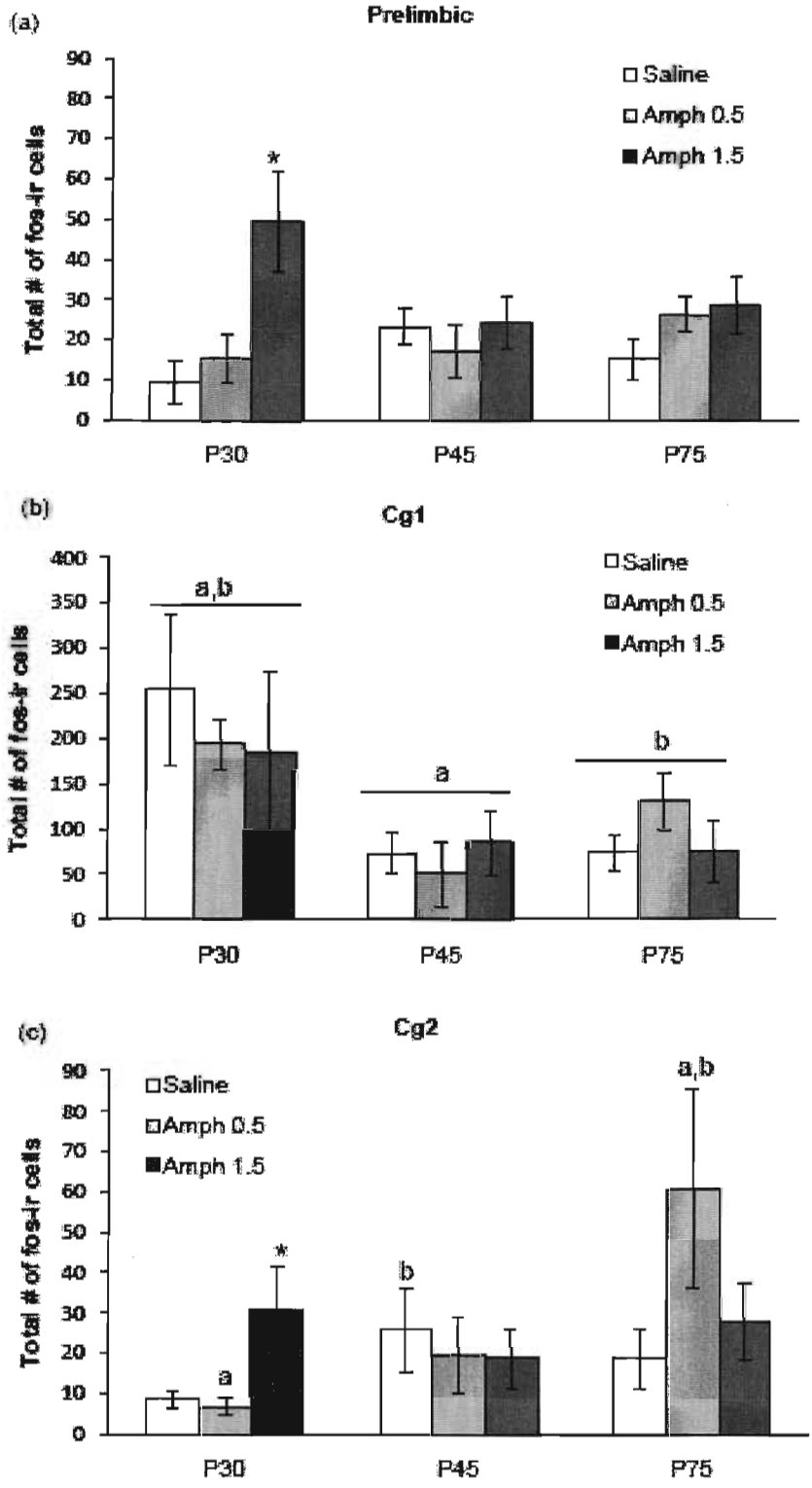

*Figure 4.4. Mean (±SEM) number of Fos-ir cell counts in the (a) prelimbic, (b) Cg1, and (c) Cg2 regions of the medial prefrontal cortex. *Amph 1.5 > Saline and Amph 0.5. Same letters indicate a significant group difference.*

Experiment 2 Discussion

As for pCREB, findings with Fos-ir are consistent with a higher overall dopaminergic tone in adolescent compared to adult rats in the NAc and the Cg1 subregion of the mPFC. Fos-ir in the NAc was somewhat more sensitive to drug effects than was pCREB, as evidenced by higher Fos-ir in rats that had 1.5 compared to 0.5 mg/kg of amphetamine. However, the difference in Fos-ir between amphetamine and saline did not reach significance, indicating that Fos-ir in the NAc alone cannot account for age differences in locomotor activity.

Although the locomotor activating effects of amphetamine necessarily involve actions in the NAc (Sellings & Clarke, 2003), amphetamine-induced activation of dopamine receptors in the mPFC also regulates locomotor activity (Hall et al., 2009; Tzschentke & Schmidt, 2000). In the present study, we found that the 1.5 mg/kg dose of amphetamine induced more Fos-ir compared to the 0.5 mg/kg dose and to saline in the prelimbic and Cg2 subregions of the mPFC only in P30 rats. The finding that the high dose of amphetamine increased Fos-ir only in P30 rats is consistent with the hypothesis that the mPFC has greater control over behaviour in adolescence than in adulthood (Ernst et al., 2009). These findings suggest that the prelimbic mPFC may be more strongly involved in regulating locomotor activity in P30 than in P45 and adult rats at the 1.5 mg/kg dose of amphetamine. Prelimbic D1 dopamine receptors are particularly relevant for locomotor activating effects of amphetamine (Hall et al., 2009), which undergo changes in density and localization in the prelimbic mPFC in adolescence (Andersen et al., 2000; Brenhouse, Sonntag et al., 2008), indicating that the prelimbic mPFC may exert

greater control over locomotor activating effects of amphetamine in P30 rats specifically through D1 dopamine receptors.

Experiment 3 was designed to directly investigate the hypothesis that prelimbic D1 dopamine receptors would contribute more to locomotor activating effects of 1.5 mg/kg of amphetamine in P30 than in older rats by injecting a D1 receptor antagonist directly into the prelimbic mPFC before a systemic injection of amphetamine. We also injected a D2 receptor antagonist into the prelimbic mPFC to investigate potential differences in contribution of these receptors to locomotor activity, as D2 receptors also undergo developmental changes in adolescence (Andersen et al., 2000).

Method Experiment 3

Surgery

Rats underwent stereotaxic surgery for bilateral cannulae implantation into the prelimbic region of the mPFC on P25, P40, or P70. Rats were anaesthetized with a ketamine/xylazine mixture and guide cannulae were implanted into the prelimbic mPFC using age-appropriate coordinates (P70: AP 12.3 from lambda, ML 0.9, and DV 1.8; P25: AP 10.9, ML 0.9, DV 0.7; P40: AP 11.5, ML 0.9, DV 1.5). Coordinates for adult rats were based on the atlas of Paxinos and Watson (Paxinos & Watson, 2005) and coordinates for the adolescent groups were determined from the initial surgeries. Guide cannulae, 13 mm in length, were constructed from 23 gauge needles and positioned 1 mm above the injection site, such that the injection needle (30 gauge, 14 mm in length) protruded 1 mm below the tip of the guide cannulae. Guide cannulae were secured in place using stainless-steel jewellery screws and dental acrylic and were plugged with

removable pins. Rats were given 5 days to recover from surgery before the start of testing.

Drug administration

47 rats were used for Experiment 3. Rats were given a bilateral injection of saline (0.5 µl/side) into the prelimbic mPFC and then were habituated to the test arena for 1 h on either P30, P45, or P75. Injections were administered over 1 min with Hamilton constant rate micro syringes (CR 700) that were connected to stainless-steel injecting needles by polyethylene (PE-10) tubing. The injection needles were removed after an additional minute to allow for diffusion of vehicle into the brain and then rats were placed into the locomotor test arena for 15 min. After 15 min, rats were given an intra-peritoneal injection of saline and were placed back into the test arena for 1 h of habituation. D1 (SCH 23390; Sigma-Aldrich) and D2 (raclopride; Sigma-Aldrich) dopamine receptor antagonists were administered in counterbalanced order over 2 test days within a dose range that was previously found to regulate drug responses when administered in the CNS (D Hall et al., 2009; Samson & Chappell, 2003). During the first test day (P31, P46, or P76), rats were given an injection of either saline (0.5 µl/side), the D1 receptor antagonist, SCH 23390 (1.0 µg in 0.5 µl of saline; SCH 1.0) or the D2 receptor antagonist, raclopride (1.0 µg in 0.5 µl of saline; Racl 1.0) into each hemisphere immediately before placement into the test arena for 15 min. After 15 min, all rats were given an intra-peritoneal injection of 1.5 mg/kg of amphetamine (Amph 1.5) and were placed back into the test arena for 1 h. The procedure was repeated 48 h later, except that animals that had raclopride on the first day had SCH 23390 on the second day and vice versa. Locomotor activity was recorded for the first 15 min after intra-mPFC drug

injection to determine potential effects of antagonists on baseline locomotor activity and for the 1 h after intra-peritoneal amphetamine injection to determine effects of antagonists on amphetamine-induced locomotor activity. Locomotor activity was recorded using a Sony video camera mounted from the ceiling and connected to a computer tracking system (Smart; Panlab, Spain) that recorded distance traveled in cm. After testing was completed, rats were given a bilateral injection of 0.5 μl of 2.5% methylene blue dissolved in saline directly into the prelimbic mPFC to confirm cannulae placements. Brains were sliced into 50 μm sections, mounted onto slides, and examined under a light microscope for localization of cannulae placements. Only rats with both injection sites terminating in the prelimbic mPFC were included in the analyses. Injection sites ranged from AP: 13.2 – 11.5; ML: 0.2 – 1.0; DV: 2.8-3.8. This resulted in the removal of 6 rats from the analysis because of incorrect cannulae placement.

Statistics

Locomotor activity was analyzed with factorial ANOVA with the between group factors of Age (P30, P45, P75) and mPFC Drug (Saline, SCH 1.0 or Racl 1.0). Given the counterbalanced design, in which the same rats were used for investigation of intra-mPFC SCH 23390 and raclopride, locomotor activity for each drug was subjected to separate factorial ANOVA after confirming that there were no order effects of drug administration. Additional analyses were conducted to estimate the effect of intra-mPFC drug injection specifically on the locomotor activating effects of amphetamine by calculating a difference score, for which activity for rats in the SCH 1.0 and Racl 1.0 groups was subtracted from activity for rats in the Saline group. The difference score for

each drug (SCH 1.0, Racl 1.0) was analyzed by one-way ANOVA with Age (P30, P45, P75) as the between-subjects factor.

Experiment 3 Results

Did the effect of intra-mPFC D1 dopamine receptor antagonist SCH 23390 depend on age?

Baseline activity. For locomotor activity during the first 15 min of testing, there was an effect of mPFC Drug ($F(1,35) = 7.49$, $p = 0.01$) only, with rats in the mPFC-SCH 1.0 group moving less than rats in the mPFC-Saline group (see Figure 4.5 a).

There was no age difference in the degree of inhibition (mPFC-Saline – mPFC SCH 1.0) produced by mPFC-SCH 1.0 (see Figure 4.5 b).

Activity after a systemic injection of 1.5 mg/kg amphetamine. Locomotor activity after amphetamine was lower for rats in the mPFC-SCH 1.0 group than for rats in the mPFC-Saline group ($F(2,35) = 41.84$, $p < 0.0001$). The interaction of Age X mPFC Drug approached significance ($F(2,35) = 3.05$, $p = 0.06$). Follow-up analyses confirmed that rats in the mPFC-SCH 1.0 group had lower activity than rats in the mPFC-Saline group at all ages (all $p < 0.02$). An age difference was not found for rats in the mPFC-Saline group. For rats in the mPFC-SCH 1.0 group, there was an effect of Age ($F(2,27) = 3.55$, $p = 0.04$): P75 were more active than P30 rats ($p = 0.02$) and there was a trend for greater activity for P75 than for P45 ($p = 0.06$) rats (see Figure 5c).

SCH 1.0 inhibited locomotor activity ($F(2,26) = 9.98$, $p = 0.001$) to a greater degree (mPFC-Saline – mPFC SCH 1.0) in P30 than in P45 ($p < 0.0001$) and P75 ($p = 0.001$) rats (see Figure 4.5 d).

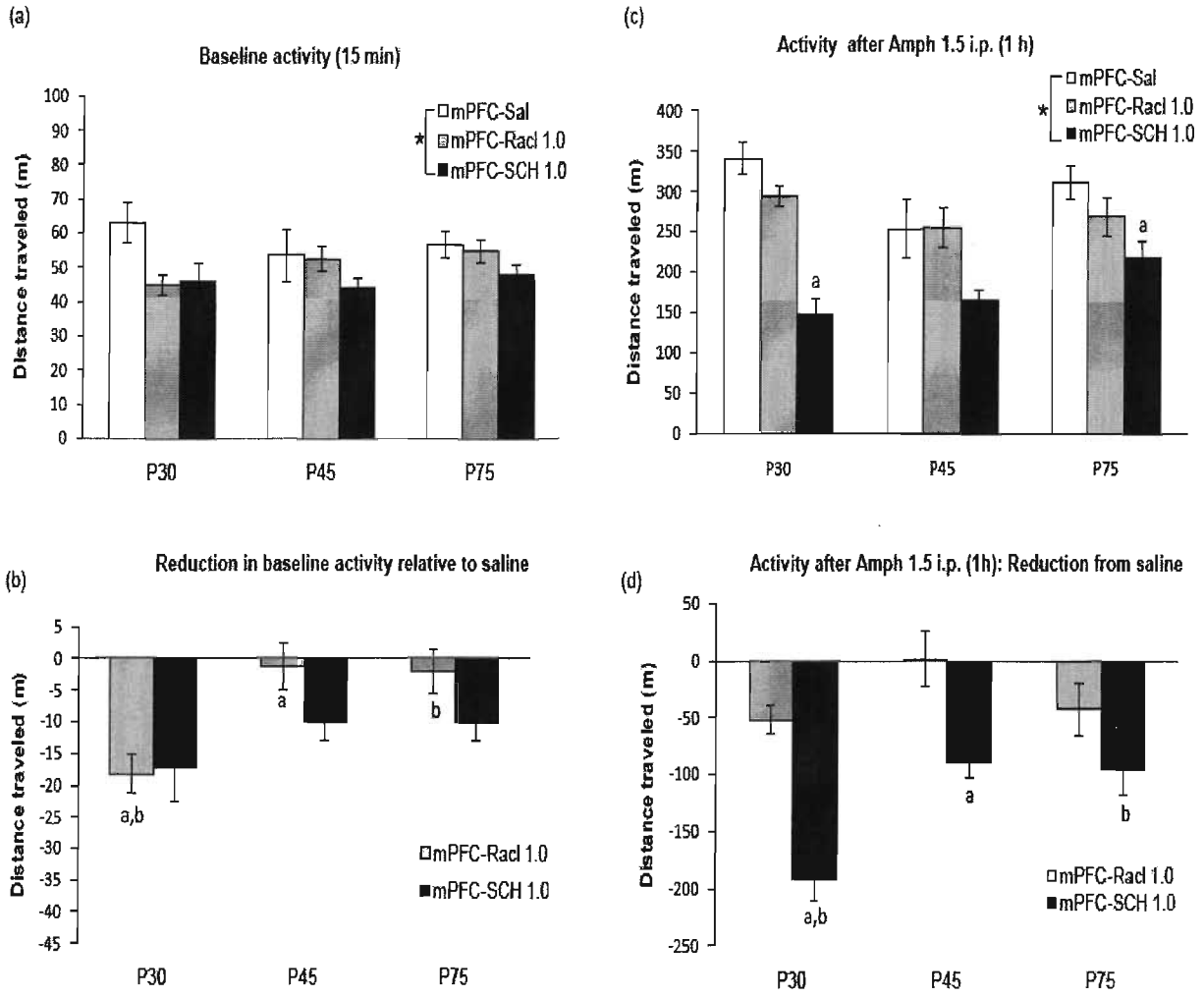

*Figure 4.5. (a) Mean (±SEM) distance traveled in a 15 min baseline (pre-amphetamine) immediately after an intra-mPFC injection of saline, the D1 antagonist SCH 23390, or the D2 antagonist raclopride; (b) Mean (±SEM) reduction in distance traveled relative to saline after administration of SCH 23390 and of raclopride in the 15 min baseline; (c) Mean (±SEM) distance traveled after i.p. injection of 1.5 mg/kg of amphetamine; (d) Mean (±SEM) reduction in distance traveled in SCH 23390 and raclopride groups relative to saline after i.p. injection of 1.5 mg/kg amphetamine. *Main effect of dose; Same letters indicate a significant group difference.*

Did the effect of intra-mPFC D2 dopamine receptor antagonist raclopride depend on age?

Baseline activity. For locomotor activity in the first 15 min after intra-mPFC injection, the effect of mPFC Drug approached significance ($F(1,35) = 3.66$, $p = 0.06$), with rats in the mPFC-Raclopride group moving less than rats in the mPFC-Saline group (see Figure 4.5 a).

Intra-prelimbic injection of raclopride ($F(2,26) = 8.09$, $p = 0.002$) produced a greater degree of inhibition in P30 than in P45 ($p = 0.001$) and P75 ($p = 0.003$) rats (see Figure 4.5 b).

Activity after a systemic injection of 1.5 mg/kg amphetamine. Raclopride did not reduce amphetamine-induced locomotor activity compared to saline at any age (see Figure 4.5 c and d).

Experiment 3 Discussion

In experiment 2, we found that the 1.5 mg/kg dose of amphetamine induced a sharp increase in Fos-ir compared to the 0.5 mg/kg dose and to saline in the prelimbic subregion of the mPFC only in P30 rats. We hypothesized that this increased activation in P30 rats reflects greater contribution of prelimbic D1 dopamine receptors to locomotor activating effects of 1.5 mg/kg amphetamine compared to older rats because prelimbic D1 receptors are thought to have greater control over behaviour in adolescence (Ernst et al., 2009) and because D1 receptors regulate locomotor activating effects of amphetamine in adults (D Hall et al., 2009). Consistent with this hypothesis, we found that an intra-mPFC injection of a D1 receptor antagonist inhibited amphetamine-induced locomotor activity to a greater extent in P30 than in P45 and adult rats, indicating that prelimbic D1

receptors are more involved in regulation of locomotor activity at the 1.5 mg/kg dose in early adolescence than in adulthood. Age differences were not found for P45 and adult rats, which suggests that the regulation of amphetamine-induced activity by prelimbic D1 dopamine receptors may stabilize by late adolescence. In addition, D2 dopamine receptors did not affect amphetamine-induced activity at any age, indicating that developmental differences in D2 receptors may be less relevant for the locomotor activating effects of amphetamine at the doses used in the present study.

The data from experiment 3 suggest that P30 rats are more dependent on D1 receptor stimulation for expression of an adult-like locomotor response to 1.5 mg/kg amphetamine. Thus, our previous observation that adolescent rats are less active than adults at the 0.5 mg/kg dose (Mathews & McCormick, 2009) may reflect reduced activation of prelimbic D1 dopamine receptors at this dose. Experiment 4 was designed to test the hypotheses that the reduced locomotor activating effects of 0.5 mg/kg of amphetamine in adolescent compared to adult rats involve reduced activation of D1 dopamine receptors, and that intra-mPFC injection of a D1 dopamine receptor agonist would enhance the locomotor activating effects of a lower dose of amphetamine in adolescent rats.

Method Experiment 4

Drug administration

54 rats were used for Experiment 4. Surgeries were conducted as in experiment 3, except that P45 rats were not included. Rats were habituated to the test arena at either P30 or P75, when all rats were given a bilateral injection of saline (0.5 µl/side) into the prelimbic mPFC. Injections were administered as in experiment 3. Rats were placed into

the locomotor test arena for 15 min. After 15 min, rats received an intra-peritoneal injection of saline and were placed back into the test arena for 1 h of habituation. On the test day (P31, P76), rats were given an injection of either saline (0.5 µl/side), D1 dopamine receptor antagonist SCH 23390 (1.0 µg in 0.5 µl of saline; SCH 1.0; Sigma) or one of two doses (0.5 or 1.0 µg in 0.5 µl of saline; SKF 0.5 and SKF 1.0, respectively) of the D1 dopamine receptor agonist SKF 81297 (Sigma; doses based on Brenhouse et al., 2008) into each hemisphere before placement into the test arena for 15 min. After 15 min, all rats were given an intra-peritoneal injection of 0.5 mg/kg of amphetamine (Amph 0.5) and were placed back into the test arena for 30 min. Locomotor activity was recorded for the first 15 min after intra-mPFC drug injection and for the 30 min after intra-peritoneal amphetamine injection as in experiment 3. Localization of cannulae placements was as in experiment 3. Only rats with both injection sites terminating into the prelimbic were included in the analyses. Injection sites ranged from AP: 13.2 – 11.5; ML: 0.2 – 1.0; DV: 2.8-3.8. This resulted in removal of 6 rats from the analysis because of incorrect cannulae placement. An additional rat was removed because of an injection problem.

Statistics

Locomotor activity was analyzed with factorial ANOVA with the between group factors of Age (P30, P75) and mPFC Drug (Saline, SCH 1.0, SKF 0.5, SKF 1.0).

Experiment 4 Results

Baseline activity (First 15 min after intra-PFC injection). For locomotor activity in the first 15 min after intra-mPFC injection, there was an effect of mPFC Drug ($F(3,39) = 6.08$, $p = 0.002$) only: Rats in the mPFC-SCH 1.0 group were less active than rats in the mPFC-Saline ($p < 0.0001$), mPFC-SKF 0.5 ($p = 0.003$), and SKF 1.0 ($p = 0.005$) groups.

Intra-mPFC injection of SKF 0.5 or SKF 1.0 did not affect baseline activity (see Figure 4.6 a).

Locomotor activity was inhibited to a greater degree (mPFC-Sal – mPFC-agonist/antagonist) for rats in the mPFC-SCH 1.0 group than for rats in the mPFC-SKF 0.5 ($p = 0.002$) and mPFC-SKF 1.0 ($p = 0.003$) groups ($F(2,30) = 7.68$, $p = 0.002$). The effect of age was not significant (see Figure 4.6 b).

Activity after a systemic injection of 0.5 mg/kg amphetamine. For locomotor activity after amphetamine, there was an effect of mPFC drug ($F(3,39) = 16.25$, $p < 0.0001$) and an Age X mPFC Drug interaction ($t(9) = 3.63$, $p = 0.005$). An age difference was found for the mPFC-Saline group ($F(1,9) = 13.18$, $p = 0.005$), with P75 rats moving more than P30 rats. An age difference was found also for rats in the SCH 1.0 group ($t(11) = 2.46$, $p = 0.03$), with P75 rats moving more than P30 rats. Age differences were not found for rats treated with SKF 0.5 of SKF 1.0 (see Figure 4.6 c).

For P30 rats, there was an effect of mPFC Drug ($F(3,18) = 21.13$, $p < 0.0001$): Rats in the mPFC-Saline group were more active than rats in the mPFC-SCH 1.0 group ($p < 0.0001$) and less active than rats in the mPFC-SKF1.0 group ($p = 0.05$). Rats in the mPFC-SCH 1.0 group were less active than rats in the mPFC-SKF 0.5 ($p < 0.0001$) and mPFC-SKF 1.0 ($p < 0.0001$) groups. For P75 rats, there was an effect of mPFC Drug ($F(3,21) = 8.67$, $p = 0.001$): Rats in the mPFC-Saline group were more active than rats in the mPFC-SCH 1.0 ($p < 0.0001$), mPFC-SKF 0.5 ($p = 0.003$), and mPFC-SKF 1.0 ($p = 0.007$) groups.

For the degree of effect produced by intra-mPFC drug treatment (mPFC-sal – mPFC D1 agonist/antagonist), there was an effect of mPFC Drug ($F(2,30) = 16.65$, $p <$

0.0001), of Age ($F(1,30) = 56.70$, $p < 0.0001$), and an interaction that approached significance ($F(2,30) = 2.78$, $p = 0.078$). Activity was inhibited to a greater degree for rats in the mPFC-SCH 1.0 group compared to rats in the mPFC-SKF 0.5 and mPFC-SKF 1.0 groups (both $p < 0.0001$). Activity was inhibited to a greater degree in adults than in adolescents for all treatment groups (all $p < 0.01$) (see Figure 4.6 d).

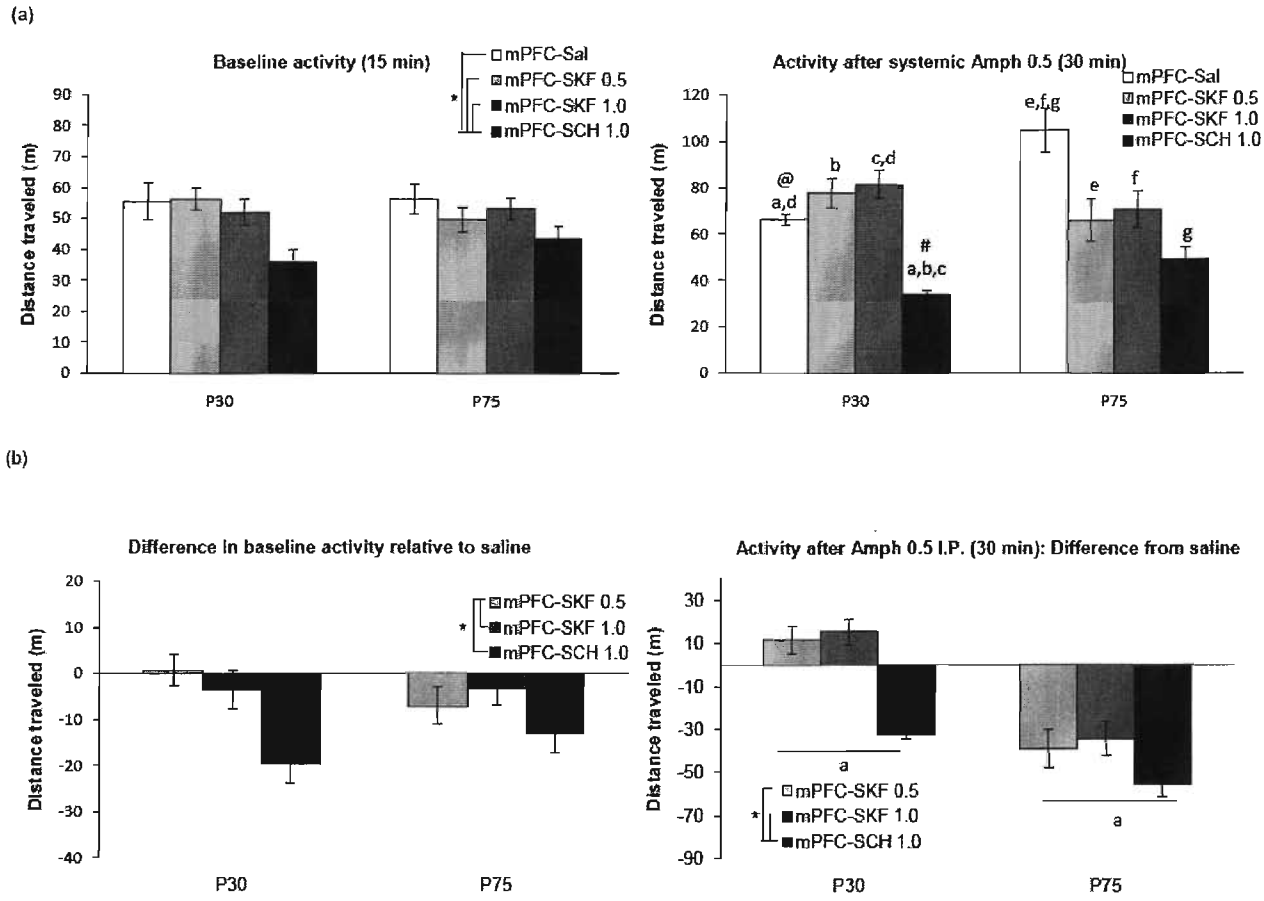

Figure 4.6. (a) Mean (±SEM) distance traveled in a 15 min baseline (pre-amphetamine) immediately after intra-mPFC injection of saline, D1 agonist SKF 81297, or D1 antagonist SCH 23390; (b) Mean (±SEM) difference in distance traveled in SKF 81297 and SCH 23390 groups relative to saline in the 15 min baseline; $^{@}P30 < P75$ ($p = 0.005$); $^{\#}P30 > P75$ ($p = 0.03$). (c) Mean (±SEM) distance traveled after i.p. injection of 0.5 mg/kg of amphetamine; (d) Mean (±SEM) difference in distance traveled in SKF 81297 and SCH 23390 groups relative to saline groups after i.p. injection of 0.5 mg/kg amphetamine.

Experiment 4 Discussion

In experiment 4, we tested two related hypotheses. First, we hypothesized that the 0.5 mg/kg dose of amphetamine would involve less activation of prelimbic D1 dopamine receptors in early adolescent than adult rats based on previous evidence for reduced locomotor activity in adolescents compared to adults at this dose (Mathews & McCormick, 2009) and based on the lack of prelimbic Fos-ir induction at this dose in P30 rats in experiment 2. Consistent with this hypothesis, injection of a D1 dopamine receptor antagonist into the prelimbic mPFC resulted in less inhibition of locomotor activity in early adolescent compared to adult rats, indicating that adolescent rats may be less active than adults at the 0.5 mg/kg dose of amphetamine in part because this dose is less effective at activating prelimbic D1 dopamine receptors in adolescence than in adulthood. In experiment 3, we found that the locomotor responding of adolescents to a 1.5 mg/kg dose of amphetamine was disrupted more by an intra-mPFC injection of a D1 receptor antagonist than was that of adult rats, which suggests that the locomotor activating effects of amphetamine are more dependent on these receptors in early adolescence than in adulthood. Thus, inadequate stimulation of D1 dopamine receptors at the low dose (0.5 mg/kg) of amphetamine in P30 rats may account for their reduced locomotor activity compared to adults.

We further hypothesized that if reduced locomotor activity at the 0.5 mg/kg dose of amphetamine can in fact be attributed to inadequate activation of prelimbic D1 receptors, then injection of a D1 receptor agonist into the mPFC of adolescent rats would increase locomotor activity in P30 rats, thereby reducing age differences in locomotor activity. This hypothesis was partially supported: An injection of the higher dose (1.0

μg/side) of a D1 receptor agonist into the prelimbic mPFC increased the locomotor activating effects of 0.5 mg/kg amphetamine in adolescent, but not in adult rats. However, rats treated with the D1 agonist did not attain as high levels of activity as did adult rats, indicating that the enhancement of locomotor activity by activation of prelimbic D1 receptors is not complete. In adults, the response to amphetamine requires additional effects on other neurotransmitter systems in the prelimbic mPFC that are also affected by amphetamine, including norepinephrine (Kuczenski & Segal, 1992) and glutamate (Del Arco, Martinez, & Mora, 1998).

In contrast to the increase in adolescents, in adult rats, injection of a D1 receptor agonist into the prelimbic mPFC reduced the locomotor activating effects of 0.5 mg/kg of amphetamine. The inhibition produced by a D1 receptor agonist was comparable to that produced by a D1 receptor antagonist. These data suggest that although D1 receptor activation is necessary for the locomotor activating effects of amphetamine in adult rats, excessive stimulation of D1 receptors also blocks the locomotor activating effects of amphetamine. Amphetamine increases dopamine release in the mPFC of adult rats (Maisonneuve, Keller, & Glick, 1990; Moghaddam & Bunney, 1989), which likely results in optimal D1 receptor stimulation for enhancement of locomotor activity, whereas further addition of the D1 agonist may result in over-stimulation of D1 receptors. This interpretation is consistent with evidence that dopamine in the prefrontal cortex has an inverted U effect on locomotor activity in adult rats (Radcliffe & Erwin, 1996), and with previous reports of inhibitory effects of intra-mPFC D1 receptor agonists on locomotor activating effects of systemic amphetamine in adult rats (Isacson, Kull, Wahlestedt, & Salmi, 2004).

General discussion

Overall, the results of present experiments demonstrate that prelimbic D1 dopamine receptors have an integral role in regulating age dependent differences in the locomotor activating effects of systemic amphetamine. We found that early adolescent rats were the only group that exhibited increased prelimbic Fos-ir after 1.5 mg/kg amphetamine. The results of D1 agonist and antagonist administration into the prefrontal cortex suggest that D1 dopamine receptors are strongly recruited to regulate behaviour at the high dose (1.5 mg/kg) of amphetamine during early adolescence, wherein these receptors contribute more to locomotor activating effects of amphetamine in early adolescent compared to late adolescent and adult rats. In contrast, a low dose (0.5 mg/kg) of amphetamine is less effective at stimulating D1 dopamine receptors in early adolescent than in adult rats, which suggests that dose-dependent age differences in locomotor activity involve differential activation of prelimbic D1 dopamine receptors at different doses.

Enhanced recruitment of D1 dopamine receptors at high doses of amphetamine may have important implications for age differences in drug addiction. Intra-mPFC injections of a D1 dopamine receptor agonist in adolescent rats increased preference for a low dose of cocaine, whereas a high dose of cocaine was sufficient on its own to produce strong place preference (Brenhouse, Sonntag et al., 2008). Moreover, prelimbic D1 dopamine receptors have been implicated in resistance to extinction of conditioned place preference and with reinstatement of drug seeking in adolescent rats (Brenhouse et al., 2010), indicating that a greater role for D1 dopamine receptors in regulation of

psychostimulant responses in adolescence may be associated with increased risk for drug dependence during this time.

Age differences between P45 and adult rats were less evident than age differences between P30 and adult rats on all measures, indicating that the dopamine system begins to show signs of maturation by late compared to early adolescence, although much maturation continues past this age for many systems (e.g., sexual maturation, reviewed in McCormick & Mathews, 2007; evident in testosterone concentrations here). Greater and more lasting effects of drug treatments have been reported when drug treatment is given in early rather than later adolescence (Mathews, Kelley & McCormick, unpublished observations). In addition, studies with people have found that dependence problems are bigger in individuals who initiated drug use in early compared to late adolescence (Clark et al., 1998; Estroff et al., 1989; Merline et al., 2004; Windle et al., 2008). Thus, stabilization of D1 receptor involvement in the regulation of psychostimulant effects by late adolescence may be associated with reduced severity of dependence problems after early adolescence.

Although the present results emphasize the importance of prelimbic D1 dopamine receptors in regulating age differences in the locomotor activating effects of amphetamine, age differences in locomotor activity also involve developmental differences in the NAc. We have previously found that adolescents are more active after intra-accumbens injections of amphetamine than are adult rats (Mathews & McCormick, 2009). In the present study, we found that pCREB and Fos-ir in the NAc are enhanced in adolescent compared to adult rats, which suggests that a higher dopaminergic tone in the

NAc during adolescence may account for altered sensitivity of this region to amphetamine during adolescence.

Conclusions

Consistent with the triadic theory of motivated behaviour, which proposes that prelimbic D1 dopamine receptors drive behaviour more in adolescence than in adulthood (Ernst et al., 2009), we have found that D1 dopamine receptors in the mPFC positively regulate locomotor activating effects of amphetamine in early adolescence to a greater extent than in adulthood. Thus, mPFC D1 receptors may be involved in enhanced risk for addiction particularly in early compared to late stage of adolescence. These findings may have implications for understanding addiction risk in people, as initiation of drug use in early adolescence is associated with more negative outcomes than initiation of drug use in late adolescence in people (Clark et al., 1998; Estroff et al., 1989; Merline et al., 2004; Windle et al., 2008).

RATIONALE FOR STUDY 4

The studies thus far have established that the ongoing development of the motivational neurocircuitry in adolescence is associated with age differences in the locomotor activating effects of acute amphetamine. Further, the finding that a rapid increase in locomotor activity to a second injection of amphetamine was observed only in adolescent rats is consistent with the perspective that adolescents are uniquely vulnerable to effects of psychostimulants. Nevertheless, it is not known from the studies thus far whether the change in sensitivity to amphetamine that was observed over the short time frame (24 h) in study 1 would result in more lasting alterations in amphetamine sensitivity in adolescent compared to adult rats, or if the enhanced sensitivity to amphetamine in adolescent rats would dissipate over time.

Study 4 was designed to test the hypothesis that two days of pre-treatment with a low (0.5 mg/kg) dose of amphetamine in early adolescence would produce a larger and a longer lasting increase in amphetamine sensitivity than would the same pre-treatment procedure in adulthood. The inclusion of both an adolescent and an adult pre-treatment group provides a test of the hypothesis that adolescence is a sensitive period of development for the lasting effects of psychostimulants.

CHAPTER 5: LOW DOSES OF AMPHETAMINE LEAD TO IMMEDIATE AND LASTING LOCOMOTOR SENSITIZATION IN ADOLESCENT, NOT ADULT, MALE RATS

Note: This section is based on the following article, with permission: Mathews, I.Z., Kelly, H., & McCormick, C.M. (2010). Low doses of amphetamine lead to immediate and lasting locomotor sensitization in adolescent, not adult, male rats. *Pharmacology, Biochemistry and Behavior,* in press.

Abstract

Although there is much evidence for age differences in behavioural responses to psychostimulants in rats, the differential, lasting impact of drug exposures has rarely been investigated using direct comparisons of adolescent and adult rats. Male rats were pre-treated with 0.5 mg/kg amphetamine or saline on either postnatal days (P) 31 and P33 or P76 and P78, and locomotor activity was measured for 1 h. Adolescent, and not adult, rats showed a significant increase in distance traveled from the first to second pre-treatment. There was no evidence of sensitization of locomotor activity in either adolescents or adults on Challenge 1 to the same dose of amphetamine when tested 12 days later on P45 (late adolescence) or on P90. Rats that were pre-treated as adolescents exhibited locomotor sensitization to 1.5 mg/kg amphetamine as adults (P60) on Challenge 2, 30 days after the initiation of pre-treatment, particularly in the group that had also received amphetamine on Challenge 1 at P45. Rats that were pre-treated as adults did not show sensitization on Challenge 2. The results suggest that the rapid adaptations to drug exposures in adolescence have greater consequences than identical treatment in adulthood, and highlight the unique vulnerability of adolescents to brief, low dose drug exposure.

Introduction

Adolescents are less sensitive than are adults to psychostimulants at first drug exposure (Weiss et al., 1994), and they transition from drug use to dependence more rapidly than do adults (Wu & Schlenger, 2003). Moreover, risk for drug abuse in adulthood is greater in individuals who initiated first use in adolescence (Merline et al., 2004), suggesting that exposure to psychostimulants in adolescence may have unique and lasting effects on sensitivity to psychostimulants in adulthood. Elevated risk for addiction in adolescence has been attributed in part to the heightened novelty seeking of adolescents (reviewed in Doremus-Fitzwater, Varlinskaya, & Spear, 2010). The neural pathways that mediate novelty-seeking, most notably the mesocorticolimbic dopamine system (reviewed in Bardo, Donohew, & Harrington, 1996), are also sites of action of psychostimulants, indicating that extensive developmental remodeling of this circuitry may underlie the differential vulnerability of adolescents and adults to drugs of abuse (e.g., Ernst et al., 2009). The changes in mesocorticolimbic circuitry in adolescence are similar in people and in rats and include changes in dopamine transporter density (people: Haycock et al., 2003; rats: Moll et al., 2000), dopamine receptor density (people: Montague, Lawler, Mailman, & Gilmore, 1999; Seeman et al., 1987; Weickert et al., 2007; rats: Andersen, 2003; Andersen et al., 1997; Andersen et al., 2000; Tarazi & Baldessarini, 2000; Tarazi et al., 1998), and tyrosine hydroxylase immunoreactivity (rats: Mathews et al., 2009; people: Weickert et al., 2007).

In rodents, adolescence begins shortly after weaning and can be divided into 3 stages (Tirelli et al., 2003): Early adolescence spans the period after weaning and before puberty, lasting from approximately postnatal day 21 (P21) to P34. Mid-adolescence

encompasses the time shortly before and after puberty and lasts from P34 to P45, with puberty (as indicated by balanopreputial separation) occurring at approximately P42 in males (reviewed in McCormick & Mathews, 2007). Late adolescence begins on P45 and lasts until P60, when rats attain sexual maturity. As in people, adolescent rodents exhibit increased levels of novelty seeking (Stansfield et al., 2004) and altered sensitivity to psychostimulants (Spear, 2000) compared to adults, indicating that studies with rodent models of adolescence can provide valuable insight into vulnerability to drug abuse in people. Consistent with age differences in psychostimulant sensitivity in people, studies with rodents find that adolescents are less sensitive to the locomotor activating effects of acute psychostimulant treatment than are adults (e.g., Adriani & Laviola, 2000; Bolanos et al., 1998; Lanier & Isaacson, 1977; Mathews & McCormick, 2007; Mathews, Morrissey, & McCormick, 2010; Mathews et al., 2009), and more sensitive to the locomotor sensitizing effects of repeated psychostimulant treatment compared to adults (Adriani et al., 1998; Laviola et al., 1999; Mathews & McCormick, 2007; Schramm-Sapyta et al., 2004). Furthermore, behavioural sensitivity to amphetamine increases rapidly in adolescence, with locomotor activity increasing significantly in response to a second injection of amphetamine 24 h after the first injection in early adolescent (~P30), but not in adult rats (Mathews et al., 2009). These latter studies involved testing confined to the adolescent period compared to the adult period, and did not address the extent to which any differences observed after adolescent treatment persist into adulthood.

The few studies that have investigated possible lasting effects of psychostimulant treatment in adolescence have involved high doses and/or long periods of pre-treatment, and rarely involved a comparison group for which pre-treatment occurred in adulthood,

which is necessary to characterize the developmental-specificity of drug effects. For example, repeated pre-treatment with a high dose of amphetamine (2.0 - 10 mg/kg) (Kolta et al., 1990; McPherson & Lawrence, 2005) or cocaine (10 – 15 mg/kg) (Marin et al., 2008; Ujike et al., 1995) in adolescence induced locomotor sensitization to a challenge dose of the drug in adulthood. Other studies have reported a sensitized response to amphetamine (Burton et al., 2010; Valvassori et al., 2007) and to cocaine (Achat-Mendes et al., 2003; Adriani et al., 2006; Brandon et al., 2001) in adulthood after chronic methylphenidate treatment in adolescence (but see Ferguson & Boctor, 2010), clearly indicating that repeated drug treatment in adolescence can induce locomotor sensitization in adulthood. Nevertheless, it is not known whether the effects described in the latter studies are unique to pre-treatment in adolescence, or if similar effects would be observed in rats pre-treated in adulthood. Studies using nicotine and methylphenidate that directly compared the effects of pre-treatment in adolescence or in adulthood suggest that lasting effects may depend on age. Whereas chronic methylphenidate treatment (2 mg/kg twice a day for 15 days) in adulthood had no effect on locomotor activity, the same pre-treatment in early adolescence (P20 to P35) reduced sensitivity to the locomotor activating effects of cocaine in adulthood (Andersen, Arvanitogiannis, Pliakas, LeBlanc, & Carlezon, 2001). In contrast, pre-treatment with nicotine (0.4 mg/kg twice a day for 7 days) in adolescence, but not in adulthood, enhanced locomotor sensitization to amphetamine 30 days later in adulthood (Collins, Montano, & Izenwasser, 2004). The latter studies suggest that lasting effects of chronic drug exposure may be greater in adolescents than in adults.

Here, we test the hypothesis that brief, low dose amphetamine exposures experienced in adolescence may be sufficient to increase the vulnerability to later drug exposures, and thus the present study involves a different approach than that of previous studies, which involved higher doses and/or more numerous pre-treatment injections. The treatment regimen we used [two injections of 0.5 mg/kg amphetamine, which falls in the therapeutic dose range for ADHD (Heijtz, Kolb, & Forssberg, 2003) and in the low to moderate dose range for enhancement of locomotor activity (Gulley et al., 2007)], was based on our previous finding that early adolescent, but not adult rats, exhibited rapid behavioural sensitization to a low dose of amphetamine after a single pre-treatment 24 h earlier (Mathews, Waters & McCormick, 2009). Here, our goal was to determine whether such rapid sensitization in adolescent rats is temporary or would the effects of such a treatment regimen be observed after much longer intervals. The expression of behavioural sensitization after pre-treatment was examined at two different time points, once in later adolescence (12 days after pre-treatment) and again in adulthood (27 days after pre-treatment). Others have found that adolescent pre-treatment with amphetamine altered locomotor sensitization to amphetamine only in adulthood and not in adolescence (P37) (Santos, Marin, Cruz, DeLucia, & Planeta, 2009). Further, we have found rats in late adolescence (>P45, <P60) to differ from both rats in early adolescence and in adulthood on behavioural responses to drugs of abuse (Mathews et al., 2009). Thus, the first challenge day for the adolescent pre-treatment group occurred in adolescence (P45), but in the post-pubertal phase. The second amphetamine challenge occurred 15 days after the first challenge, when the rats were adults (P60). The second challenge involved a higher dose of amphetamine (1.5 mg/kg) to improve the likelihood of detecting pre-

treatment effects 27 days after pre-treatment, as the use of high challenge doses facilitates expression of sensitization when a low pre-treatment dose is used (Kuczenski & Segal, 2001). Lastly, to test for the developmental specificity of the pre-treatment regimen, a group of rats underwent pre-treatment in adulthood, and was tested for behavioural sensitization after the same intervals (12 and 27 days) as those rats pre-treated in adolescence. Although others have found behavioural sensitization to amphetamine in the 0.5 – 0.6 mg/kg dose range in adult rodents using numerous drug treatments (DA Hall, Stanis, Avila, & Gulley, 2008; Kelsey & Grabarek, 1999), we predicted that the use of a brief pre-treatment period that is known to have different effects on sensitization of adolescent and adult rats in the short-term (Mathews et al., 2009) would also reveal age differences in long-lasting sensitization.

Method

Animals

Male Long Evans rats were purchased from Charles River Laboratories (St. Constant, QC, Canada) and arrived at the colony on either postnatal day 22 (P22; N = 34) or P60 (N = 34). Rats were housed in pairs and maintained on a 12h light-dark cycle with lights on at 0800h. Use of animals was approved by the Brock University Animal Care and Use Committee and followed Canadian Council on Animal Care and National Institutes of Health guidelines.

Locomotor activity testing

Locomotor testing was conducted in four white open top melamine arenas (58 cm X 58 cm X 58 cm) under indirect red light illumination to reduce anxiety associated with bright lighting. On P30 or P75, rats received an intra-peritoneal injection of saline and

were immediately placed into the test arena for 1 h of habituation The pre-treatment phase began the next day and rats were randomly assigned to receive 0.5 mg/kg of amphetamine (n = 16 at each age) or saline (n = 18 at each age) immediately before placement into the locomotor test arenas for 1 h on each of two pre-treatment days, 48 h apart. Locomotor activity during the test sessions was recorded with a Sony digital video camera mounted from the ceiling and connected to the Smart tracking system (Smart; Panlab; Spain) that measured horizontal distance traveled. The first challenge day (Challenge 1) took place 12 days after pre-treatment on either P45 or P90 (see Table 5.1 for the experimental design).

Table 5.1.

Experimental design.

Age of testing for different pre-treatment groups				
Adolescents	P31 // P33	//	P45	// P60
Adults	P76 // P78	//	P90	// P105
Saline pre-treatment	Saline (S) n = 18 (SS)		S: n = 12 (SSS) A: n = 6 (SSA)	S: n = 6 (SSSS) A: n = 6 (SSSA) A: n = 6 (SSAA)
Amphetamine pre-treatment	Amphetamine (A) n = 18 (AA)		S: n = 8 (AAS) A: n = 8 (AAA)	A: n = 6 (AASA) A: n = 6 (AAAA)
Phases of the experiment	Induction (0.5 mg/kg)		Challenge 1 (0.5 mg/kg)	Challenge 2 (1.5 mg/kg)

For Challenge 1, rats in each drug pre-treatment group were assigned to receive either saline or 0.5 mg/kg of amphetamine. The second challenge (Challenge 2) took place another 15 days later when all rats were adult (either P60 or P105). Rats from each age at pre-treatment group were further divided into five groups: Rats that received saline during pre-treatment and saline on the first challenge day received either saline (SSSS group) or 1.5 mg/kg amphetamine (SSSA group) for Challenge 2, and rats that received saline during the pre-treatment phase and 0.5 mg/kg amphetamine on the first challenge day received 1.5 mg/kg amphetamine (SSAA) on Challenge 2. Rats that were treated with 0.5 mg/kg amphetamine during pre-treatment and saline on challenge 1 received 1.5 mg/kg amphetamine (AASA) on Challenge 2. The final group of rats received amphetamine at all time points: 0.5 mg/kg during pre-treatment and on Challenge 1 and 1.5 mg/kg on Challenge 2 (AAAA) (see Table 1 for the experimental design). Each rat was always tested in the same arena. All testing occurred between 0900h and 1700h and time of testing was counterbalanced across groups.

Statistics

Analyses consisted of mixed-factor ANOVA for pre-treatment days and between-groups ANOVA for Challenge 1 and 2. Follow-up analyses for within-group comparisons were conducted using paired-samples t-test, and for between group comparisons, follow-up analyses consisted of Fisher's least significant difference (LSD) test. Alpha level for statistical significance was set at $p < 0.05$, two-tailed, however, tests of a priori hypotheses with alpha levels of $p < 0.10$ two-tailed are noted.

Results

Locomotor activity in the pre-treatment phase

A Pre-treatment Day (Pre-treatment 1, Pre-treatment 2) X Pre-treatment drug group (SS, AA) X Age (P30, P75) ANOVA on distance traveled found a significant Pre-treatment Day X Pre-treatment drug group interaction ($F(1,64) = 12.30$, $p = 0.001$) and a near significant Pre-treatment Day X Age X Pre-treatment drug group interaction ($F(1,64) = 3.75$, $p = 0.057$). Follow up analyses were conducted by age to test the hypothesis that activity would increase from the first to the second pre-treatment in adolescent, but not in adult rats. For adolescent rats, a Pre-treatment Day X Pre-treatment Group ANOVA revealed a significant interaction ($F(1,32) = 10.11$, $p < 0.01$): For rats treated with saline, distance traveled decreased from the first to the second pre-treatment ($p = 0.01$) and for rats treated with amphetamine, distance traveled increased from first to second amphetamine pre-treatment ($p = 0.04$). In adulthood, there was no change in distance traveled for either saline or amphetamine treated rats (see Figure 5.1).

For saline treated rats, adolescents were significantly less active than were adults during the first ($p = 0.05$) and second ($p < 0.0001$) day of pre-treatment, whereas the age difference between adolescent and adult rats after amphetamine treatment approached significance only during the first day of pre-treatment ($p = 0.065$; adolescent < adult) (see Figure 5.1).

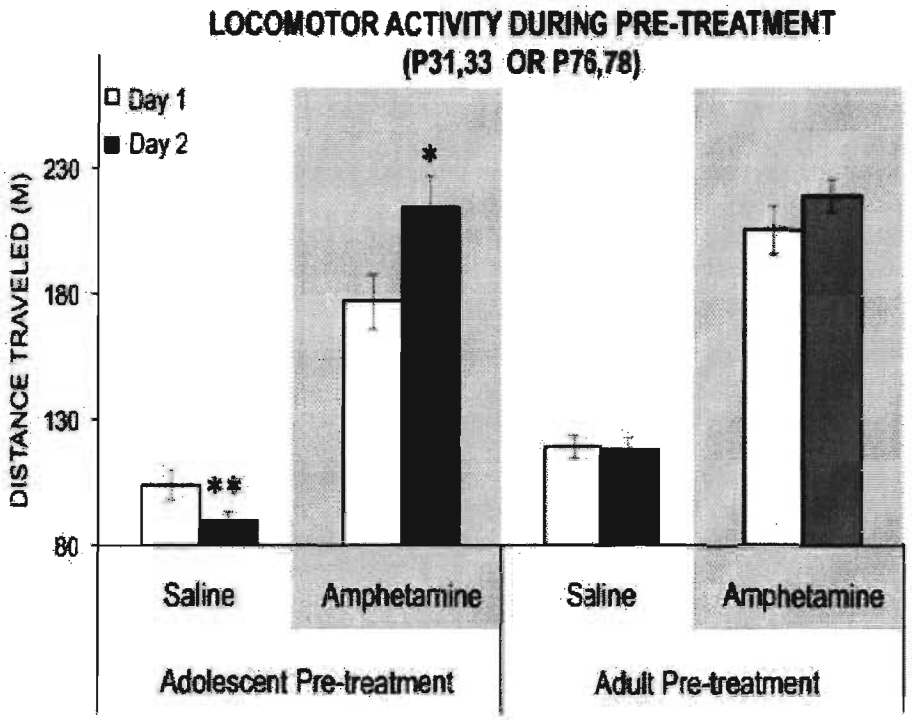

*Figure 5.1. Mean (±SEM) distance traveled during the two days of pre-treatment with saline or 0.5 mg/kg of amphetamine in adolescent or adult rats. Locomotor activity after amphetamine is shown in shaded bars. *p = 0.04 compared to activity on first pre-treatment day in amphetamine-treated adolescents; **p = 0.01 compared to activity in on first pre-treatment day in saline-treated adolescents.*

Locomotor activity in Challenge 1

An Age X Pre-treatment Drug X Challenge 1 Drug ANOVA on distance traveled on Challenge 1 revealed a main effect of Age ($F(1,60) = 11.43$, $p = 0.001$; adolescent < than adult) and a main effect of Challenge 1 Drug ($F(1,60) = 122.83$, $p < 0.0001$), with amphetamine treated rats more active than saline treated rats irrespective of pre-treatment drug. This pattern of results did not change when the analyses were conducted for each age group separately (see Figure 5.2).

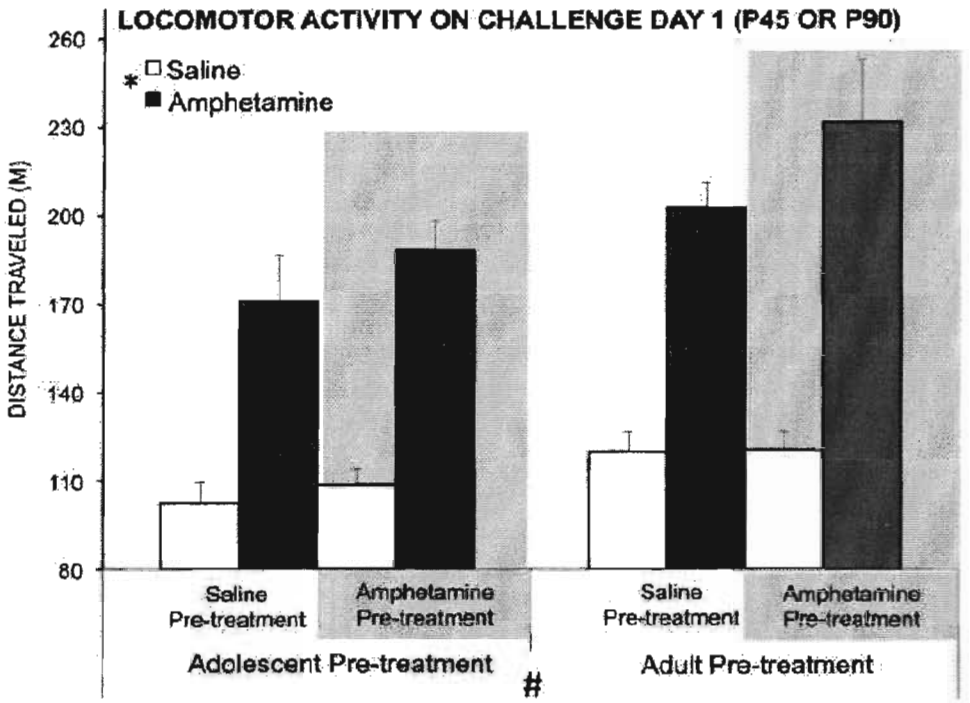

*Figure 5.2. Mean (±SEM) distance traveled after treatment with saline or 0.5 mg/kg of amphetamine on Challenge 1, 12 days after pre-treatment. Locomotor activity after amphetamine is shown in shaded bars. *main effect of Challenge 1 drug treatment ($p < 0.0001$). #main effect of age ($p = 0.001$).*

Locomotor activity in Challenge 2

Because Challenge 2 did not involve a balanced design, the data for Challenge 2 were analyzed with two different approaches. The first approach considered the five Challenge 2 groups (SSSS, SSSA, SSAA, ASAA, AAAA) as levels of one-factor. For rats pre-treated in adolescence ($F(4,29) = 15.44$, $p < 0.0001$), all rats that received amphetamine for Challenge 2 were more active than rats that received saline (all $p < 0.0001$). History of amphetamine treatment also had a significant effect on locomotor activity after an amphetamine challenge, such that rats that received amphetamine during pre-treatment and Challenge 1 (AAAA) were more active than both saline pre-treatment groups, whether or not they had amphetamine on Challenge 1 (SSSA, $p = 0.04$; SSAA, p

= 0.02). The higher activity of AAAA rats than AASA rats missed significance (p = 0.07). For adult pre-treated rats ($F(4,29) = 10.33$, $p < 0.0001$), rats in all four groups that received amphetamine for Challenge 2 were more active than the group that received saline (all $p < 0.0001$), but no other group difference was significant (see Figure 5.3).

The second approach was to have a balanced design for statistical analysis by removing the group treated with saline throughout the experiment (SSSS). A Pre-treatment drug group X Challenge 1 drug group ANOVA of distance traveled in Challenge 2 for rats pre-treated in adolescence found that the higher locomotor activity in rats pre-treated with amphetamine compared to rats pre-treated with saline did not reach statistical significance ($F(1,24) = 3.59$, $p = 0.07$). No group difference approached significance for rats pre-treated as adults (see Figure 5.3).

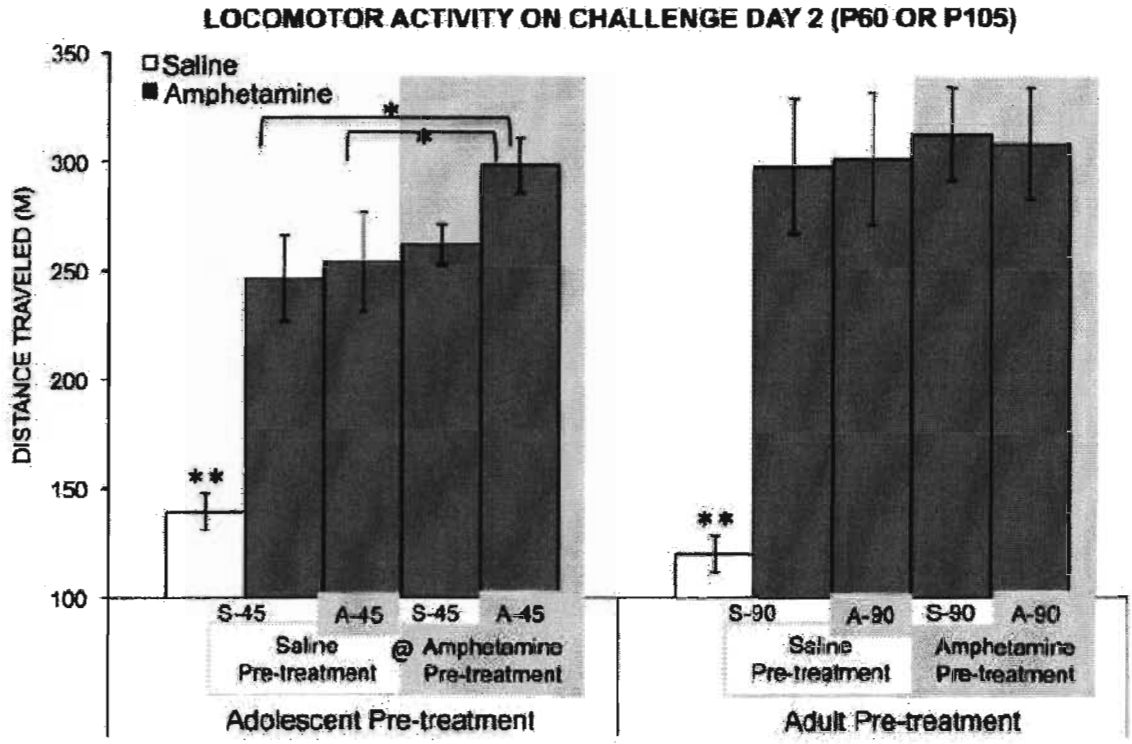

*Figure 5.3. Mean (±SEM) distance traveled after treatment with saline or 1.5 mg/kg of amphetamine on Challenge 2. Groups denote assignment during pre-treatment to saline or amphetamine and during Challenge 1 to treatment with saline (Adolescents: S-45; Adults: S-90) or amphetamine (Adolescents: A-45; Adults: A-90). Amphetamine pre-treatment groups are shown in shaded bars. *Denotes a significant difference compared to specified pre-treatment and Challenge 1 group ($p < 0.05$). @Denotes difference between rats that were pre-treated with amphetamine vs. saline in adolescence or in adulthood, irrespective of treatment on Challenge 1 ($p = 0.07$).*

Discussion

The main finding of this study is that a brief treatment regimen with low doses of amphetamine in early adolescence leads to a lasting change in response to subsequent exposures, and highlight the heightened sensitivity of adolescents compared to adults to drug-induced behavioural plasticity. First, a low dose of amphetamine in adolescence, not in adulthood, increased the locomotor activating effects of a second injection of

amphetamine given 48 h later. Second, the rapid plasticity observed during the two days of pre-treatment in adolescence was associated with long-lasting locomotor sensitization to amphetamine, whereas pre-treatment was without effect on later responses to amphetamine in adults. Third, the enhanced sensitization observed in response to the second amphetamine challenge in adulthood can be attributed primarily to effects of amphetamine pre-treatment during early adolescence, though a combinatorial effect of amphetamine treatment during early and late adolescence seems to be the basis for the effect. That the same treatment regimen in adult rats had no lasting effect on locomotor activating effects of amphetamine is consistent with adolescence as a unique period of sensitivity to enduring effects of psychostimulants. Each of these findings and their implications are discussed in greater detail below.

Locomotor activity during pre-treatment

Consistent with previous reports of hyporesponsivity to an initial exposure to psychostimulants in adolescence (Adriani & Laviola, 2000; Bolanos et al., 1998; Lanier & Isaacson, 1977; Mathews & McCormick, 2007; Mathews et al., 2010; Mathews et al., 2009), adolescent rats were less active than were adults to the first dose of amphetamine during the pre-treatment phase. In addition, only adolescent rats exhibited a significant increase in the locomotor activating effects of amphetamine on the second pre-treatment day, confirming our previous report of rapid amphetamine-induced behavioural plasticity in adolescence (Mathews et al., 2009). The increased activity in adolescents on the second test day eliminated the age differences that were observed for the first amphetamine treatment, indicating that the initial hyporesponsiveness cannot be attributed to age differences in amphetamine pharmacokinetics or to reduced locomotor

capacity in adolescents than in adults. Other studies have found that pharmacokinetic factors do not account for age differences in locomotor activity, in that brain levels of amphetamine, cocaine, or methamphetamine could not account for age differences in locomotor activity (Frantz et al., 2006; Spear & Brake, 1983; Zombeck et al., 2009). Furthermore, we have found age differences to acute administrations of a range of doses of amphetamine administered via cannulae directly into the nucleus accumbens (Mathews & McCormick, 2009). In addition, the increase in activity to a second treatment of amphetamine found in the present study cannot be attributed to a nonspecific increase in basal activity in adolescence, because adolescent saline-treated rats traveled less on the second than on the first day of pre-treatment. We have argued that the rapid change in the locomotor response to amphetamine in adolescence may reflect greater plasticity in the mesolimbic circuitry in adolescents than in adults (Mathews et al., 2009), which is consistent with the report of a lower release of striatal dopamine in adolescent than in adult rats after acute treatment of amphetamine and a higher release in adolescent rats than in adult rats after repeated treatment of amphetamine (Laviola et al., 2001).

Locomotor activity on Challenge day 1

Adolescent and adult pre-treated rats were tested for locomotor sensitization to 0.5 mg/kg of amphetamine 12 days after pre-treatment (Challenge 1), when the adolescent group was post-pubertal (P45). Locomotor sensitization was not observed in either adolescent or adult rats at this time point, although activity appeared to be slightly higher in rats of both age groups that were pre-treated with amphetamine compared to the age-matched groups pre-treated with saline. In addition, we found that adolescent rats were less active than were adults after the amphetamine challenge on P45. We previously

found that, in contrast to P30 rats which attained adult-like levels of activity to a second treatment of amphetamine, P45 rats remained hyporesponsive (Mathews et al., 2009), indicating that there are significant developmental shifts in drug responses within the time span conventionally considered as adolescence. Early and late adolescent rats differ on various behavioural and neural parameters, including conditioned place preference (Badanich et al., 2006; Brenhouse, Sonntag et al., 2008), cocaine-induced locomotor activity (Badanich et al., 2008), cocaine-induced dopamine release in the nucleus accumbens (Badanich et al., 2006), tyrosine hydroxylase immunoreactivity in the caudate nucleus (Mathews et al., 2009) and dopamine receptor expression throughout the mesocorticolimbic dopamine system (Andersen et al., 1997; Andersen et al., 2000). Thus, lower locomotor activity in P45 rats compared to adult rats is likely a reflection of a developmental shift in neural regions that regulate the locomotor activating effects of psychostimulants.

One possible explanation for the lack of sensitization at this age in adolescent pre-treated rats is that the strong developmental difference in locomotor activity on P45 masked any enhancement of activity that may have been produced by amphetamine pre-treatment on P30. Others have shown that cross-sensitization to amphetamine after 7 days of nicotine pre-treatment in early adolescence was not found if the challenge test also occurred in adolescence, but it was found if the challenge test occurred in adulthood (Santos et al., 2009). Similarly, effects of MDMA pre-treatment in adolescence on locomotor sensitization to cocaine increased with longer delays between pre-treatment and challenge day (Achat-Mendes et al., 2003). Twice daily pre-treatment with 0.5 mg/kg of amphetamine from P22 to P34 also failed to produce sensitization in rats when test for

sensitization occurred in late adolescence (~P48) (Heijtz et al., 2003), but this study did not involve additional testing in adulthood. Thus, that changes induced by drug treatment in adolescence may be dormant until adulthood, is consistent with our results for Challenge 2 (discussed below). Another explanation for the lack of sensitization on Challenge 1 may be that the dose of amphetamine used on the first challenge day was not sufficiently high to reveal sensitization, as the use of low doses of amphetamine for pre-treatment and challenge sessions may compromise the ability to detect sensitization (Kuczenski & Segal, 2001).

Locomotor activity on challenge day 2

Adolescent and adult pre-treatment groups were challenged with 1.5 mg/kg of amphetamine on either P60 or P105, 27 days after pre-treatment. At this time point, locomotor sensitization was observed only in rats that were pre-treated with amphetamine in adolescence, indicating that enhanced plasticity observed after acute amphetamine treatment in adolescence produced effects that persisted into adulthood. Even though sensitization was greatest in rats that received amphetamine both during the pre-treatment period in early adolescence and on the first challenge day on P45, a trend for sensitization was also found on P60 for those that received amphetamine on P30 and saline on P45, indicating that early adolescence (~P30) may represent a unique window of vulnerability to the effects of amphetamine. In contrast, rats given amphetamine for the first time on P45 (Challenge 1) did not differ on P60 from rats given amphetamine for the first time on P60, indicating that a single dose of amphetamine in late adolescence is not sufficient to induce locomotor sensitization 15 days later.

Most studies of the long-lasting effects of psychostimulant treatment in adolescence have used methylphenidate (Achat-Mendes et al., 2003; Brandon et al., 2001; Burton et al., 2010), and those that have examined lasting effects of amphetamine (Kolta et al., 1990; McPherson & Lawrence, 2005) and cocaine (Marin et al., 2008; Ujike et al., 1995) have used high doses (2 - 10 mg/kg of amphetamine) and prolonged pre-treatment periods. Here, we show that three days of low-dose amphetamine in adolescence are sufficient for inducing sensitization in adulthood. This is an important point to consider because many studies have reported age differences in the locomotor activating effects of acute psychostimulant treatment (Badanich et al., 2008; Bolanos et al., 1998; Lanier & Isaacson, 1977; Mathews & McCormick, 2007; Mathews et al., 2010; Mathews et al., 2009), but the age-specific impact of acute psychostimulant treatment on subsequent drug responses has not been thoroughly investigated. We found that acute amphetamine in adolescence contributed to rapid and enduring alterations in amphetamine sensitivity, as demonstrated by enhanced activity on the second pre-treatment day and on the final challenge day.

There is a lack of studies that directly compare the effects of amphetamine pre-treatment in adolescence and in adulthood. A crucial advantage of including an adult pre-treatment group is the ability to draw conclusions regarding effects that are unique to the developmental period at which the treatment occurred. Evidence for adolescence as a unique period of sensitivity during which exposure to various environmental stimuli can alter vulnerability to drugs of abuse is growing. Previous work from our lab has shown that exposure to a mild chronic social stressor throughout adolescence, but not in adulthood, increases locomotor sensitization to amphetamine in adulthood (Mathews et

al., 2008; McCormick et al., 2005). Results of the present study extend these findings by demonstrating that three exposures to a relatively low dose of amphetamine administered during adolescence can also produce lasting effects on subsequent responses to amphetamine in adulthood. These data do not suggest that sensitization does not develop in adulthood. In fact, even a single pre-treatment with a high dose of 5.0 mg/kg amphetamine has been shown to produce locomotor sensitization that increased with longer periods of withdrawal in adult rats (Vanderschuren & Kalivas, 2000). Instead, our data highlight the differential sensitivity of adolescents than adults for developing sensitization, even when exposure involves few treatments at low doses. One limitation of such developmental comparisons is that even though all rats have reached adulthood by the final test day, there is nonetheless a 45 day age discrepancy that confounds direct comparisons of locomotor sensitization because of differences in basal activity between young (P60) and older (P105) adults. For this reason, it is critical that conclusions regarding age-specific pre-treatment effects on locomotor sensitization are limited to comparisons of age-matched controls, for which baseline activity is not an issue.

Conclusion

Comparable to age differences in drug effects in people (Weiss et al., 1994), adolescent and adult rats differ in sensitivity to initial treatment with amphetamine. Importantly, acute amphetamine treatment produces adaptations that increase sensitivity to subsequent amphetamine treatment more readily in adolescent rats than in adults, indicating that very few low-dose amphetamine exposures in adolescence can have lasting consequences even though identical pre-treatment in adulthood has no detectable effect. Enhanced sensitization after adolescent pre-treatment in adulthood suggests that

adolescence, particularly the early period, may represent a unique window of vulnerability to lasting effects of psychostimulants.

CHAPTER 6: GENERAL DISCUSSION

The aim of this dissertation was to better understand how age differences in sensitivity to amphetamine map on to the development of the mesocorticolimbic dopamine system in adolescence. Locomotor activity was selected as the primary measure of sensitivity to amphetamine because the expression of amphetamine's locomotor activating effects requires coordinated activation of regions within the motivational neural circuit that are also involved in regulating the reinforcing effects of drugs (e.g., Pierce & Kalivas, 1997; Wise & Bozarth, 1987). Thus, acute amphetamine treatment can be used to elucidate age differences in the initial sensitivity of these underlying neural structures, whereas repeated amphetamine treatment can be used to draw inferences about developmental differences in neural plasticity. In addition, localized intracerebral injections of amphetamine and of dopamine receptor agonists and antagonists can provide important insights into the changing relationship between specific neural structures and behaviour as the organism develops.

Model of age differences in sensitivity to amphetamine

My investigation of the locomotor activating effects of amphetamine in relation to the development of the nucleus accumbens and the medial prefrontal cortex suggest the following model of the neural basis for age differences in amphetamine sensitivity (see Figure 6.1). The mesocorticolimbic dopamine system is in a hypoactive state during adolescence that can be overcome either with a higher dose (1.5 mg/kg), or with repeated injections of a lower dose (0.5 mg/kg), of amphetamine. The fact hypoactivity can be overridden suggests that age differences in amphetamine sensitivity may reflect a subtle increase in the response threshold of the nucleus accumbens and the medial prefrontal

cortex in adolescence. In addition, my finding of increased locomotor activating effects of intra-accumbens amphetamine in adolescent compared to adult rats suggests that hypoactivity to systemic amphetamine may involve a heightened influence of inhibitory brain regions outside of the nucleus accumbens in adolescence.

The nucleus accumbens

Locomotor hypoactivity was observed to the 0.5 and not to the 1.5 mg/kg dose of amphetamine (studies 1, 3, & 4), which suggests that the amount of stimulation required to activate the nucleus accumbens is elevated during adolescence. The elevated response threshold may be attributed to the reduced availability of dopamine pre-synaptically. Pre-synaptically, reduced levels of basal tyrosine hydoxylase, the rate limiting enzyme in the synthesis of dopamine (study 1), and of dopamine (Badanich et al., 2006) in adolescent rats may result in reduced dopamine release in response to lower doses of amphetamine. In fact, there is a report of lower dopamine release in response to acute amphetamine treatment in the caudate nucleus of adolescent rats (Laviola et al., 2001). Post-synaptically, higher expression of pCREB (Study 2), cAMP (Andersen, 2002), and dopamine D1 and D2 receptors (Andersen et al., 2000; Tarazzi & Baldessarini, 2000), as well as a reduced ability of dopamine receptors to regulate cAMP (Andersen, 2002) may make it more difficult to distinguish weak signals from the high levels of "noise" in the nucleus accumbens during adolescence.

Inhibitory regions

Reduced sensitivity to a low dose of systemic amphetamine may also involve inhibitory effects of regions outside of the nucleus accumbens, because adolescent rats were more active than were adults when amphetamine was administered directly into the

accumbens (study 2). A previous study has found that the hippocampus has a particularly strong inhibitory effect on the locomotor activating effects of amphetamine in adolescence, which was reversed by hippocampal lesions before the treatment with amphetamine (Lanier & Isaacson, 1977). Further, it has been hypothesized that the development of the amygdala may affect drug responses in adolescence (Ernst et al., 2009). In adults, the amygdala also has an inhibitory effect on amphetamine-induced locomotor activity (Woods & Ettenberg, 2004) but the extent to which this inhibitory influence may change over adolescence has not been investigated.

The medial prefrontal cortex

My research indicates that activation of prelimbic D1 dopamine receptors may be a key factor in overcoming hypoactivity when the receptors are sufficiently stimulated, as with the 1.5 mg/kg dose of amphetamine. Consequently, hypoactivity at the 0.5 mg/kg dose may involve reduced stimulation of prelimbic D1 dopamine receptors in adolescence, as evidenced by increased activity after treatment with a D1 receptor agonist (Study 3). The higher threshold for activation of D1 dopamine receptors in the prelimbic mPFC may involve similar developmental factors as in the nucleus accumbens, including higher D1 receptor density (Andersen et al., 2000), reduced D1 receptor coupling to cAMP (Andersen, 2002), lower levels of tyrosine hydroxylase (Study 1) and reduced density of dopaminergic inputs from the ventral tegmental area (Kalsbeek et al., 1988) in adolescent compared to adult rats. Moreover, the prelimbic mPFC is a major source of glutamatergic projections to the nucleus accumbens (Porrino & Lyons, 2000), which are regulated by prelimbic D1 dopamine receptors (Brenhouse et al., 2008). The density of these projections is lower in adolescent compared to adult rats (Brenhouse et al., 2008),

indicating that higher levels of D1 dopamine receptor activation may be required for the effective utilization of the available projections.

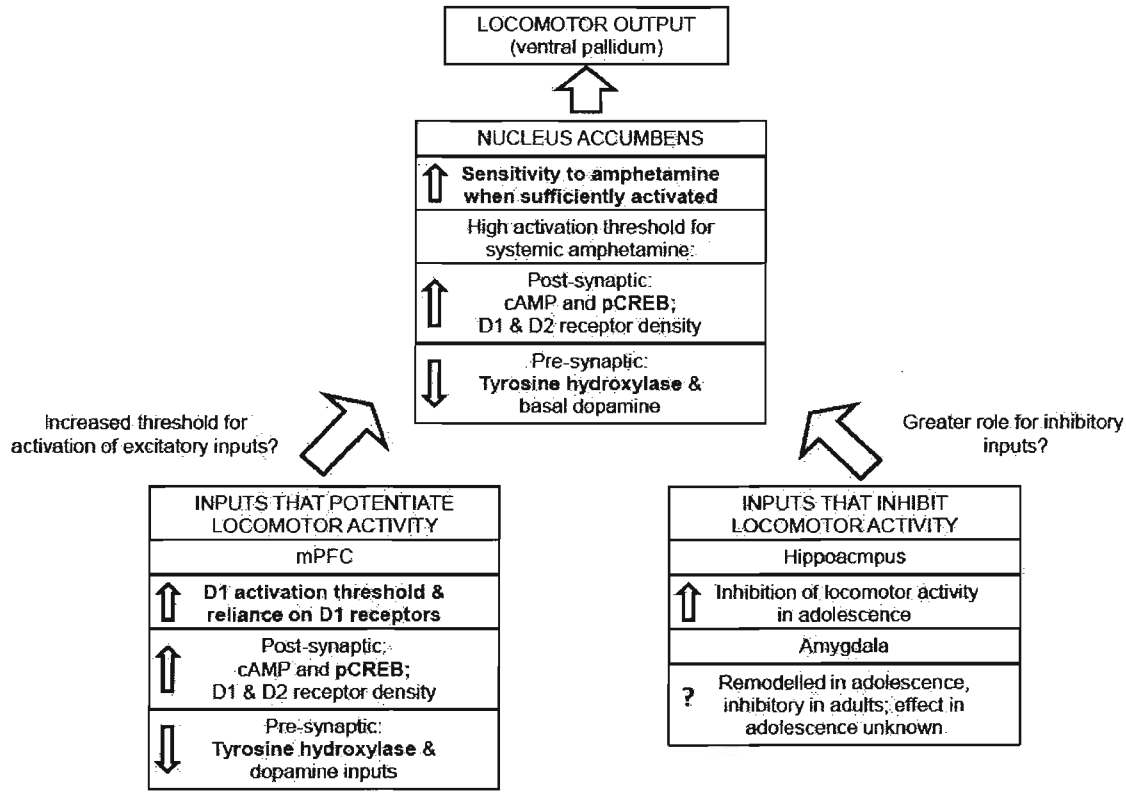

Figure 6.1. Graphical representation of findings from the thesis (bold) and from the literature that are relevant for explaining age differences in locomotor activating effects of amphetamine. Arrows within each box refer to the direction of the difference in adolescents compared to adults.

Implications for drug abuse and addiction in adolescence

Findings from the thesis research have implications for understanding the altered risk for drug abuse and addiction in adolescence. Prelimbic D1 dopamine receptors are implicated in the development of addiction through potentiation of drug-seeking behaviour and reinstatement of drug-seeking after a period of abstinence (Everitt & Wolf, 2002; Kalivas, Volkow, & Seamans, 2005; Sanchez et al., 2003; Graham, Happenot, Hendryx, & Self, 2007; Brenhouse et al., 2010). In adolescence, my results indicate that

low doses of amphetamine have insufficient action at prelimbic D1 dopamine receptors to increase locomotor activity to levels comparable to adults. Thus, in adolescents, low doses have less of an effect on behaviour, which may increase the likelihood of repeated usage (e.g., Laviola et al., 1999; Weiss et al., 1994), thereby increasing the possibility of the drug-related changes in plasticity that underlie drug abuse. The results also indicated that although a higher dose resulted in the same level of activity in adolescents and adults, the locomotor response in adolescent rats reflected proportionally greater involvement of prelimbic D1 receptors compared to adult rats. The latter results suggest that the same dose of amphetamine may lead to higher levels of drug-seeking in adolescent than in adult rats, which is consistent with the enhanced risk for addiction in adolescence in people (reviewed in Spear, 2000).

The results from study 4 also highlight differential consequences of drug exposure in adolescents than in adults. In study 4, a short period of pre-treatment with a low dose of amphetamine resulted in longer lasting locomotor sensitization than did the same pre-treatment protocol in adulthood, which is consistent with reports of faster transition from drug abuse to dependence during adolescence in people (Clark et al., 1998). Overall, the findings of the present studies are consistent with the hypothesis that adolescence is a sensitive period of development for the effects of psychostimulants.

Final comments

Psychostimulants like amphetamine act on the same neural systems that underlie motivation for natural rewards (Mogensen et al., 1980). These systems direct attention to, and promote the approach toward, stimuli that are required for adaptation and survival. Reorganization of receptors and synapses during sensitive periods of development

prepares the organism to meet the changing demands of each stage of development (e.g., Ernst et al., 2009). For example, the motivational system in neonates is highly tuned toward the dam because of the high reliance on the dam for pup survival (Shair, 2007). As pups approach adolescence, motivational circuits are again reorganized to meet the changing demands of survival in unfamiliar environments away from the dam. The challenge of exploring greater territories and encountering new conspecifics is met by increased levels of risk taking, novelty seeking, and play behaviour during adolescence (Wahlstrom et al., 2010; Spear, 2000). As rats approach adulthood, the demand for reproductive success leads to an increased interest in the opposite sex and a concomitant increase in inter-male aggression as they become competitors for territory and access to females (Thor & Carr, 1979).

Developmental fine-tuning of the motivational system suggests that even when similar behavioural outcomes are observed, the underlying neural mechanisms may nonetheless differ with age. For example, the transient expression of parental behaviour in pre-pubertal adolescent rats (reviewed in Nephew, Lovelock, & Bridges, 2008) involves different neural and endocrine mechanisms than does the same behaviour in adults. The main implication of drugs acting on developmentally-distinct neural mechanisms is the potential to permanently alter the developmental trajectory of the underlying neural substrates, as exemplified by the age-specific development of locomotor sensitization in study 4 of the thesis.

In conclusion, the same systems that promote resilience by supporting successful negotiation of developmental challenges can increase the engagement in risky behaviours that can lead to more severe maladaptive outcomes during adolescence.

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List of abbreviations

Amph 0.5	0.5 mg/kg dose of systemically administered amphetamine
Amph 1.5	1.5 mg/kg dose of systemically administered amphetamine
Cg1	Cingulate cortex 1; subregion of medial prefrontal cortex
Cg2	Cingluate cortex 2; subregion of medial prefrontal cortex
CREB	Cyclic AMP response element binding protein
i.p.	Intraperitoneal route of systemic drug administration
pCREB	Phosphorylated cyclic AMP response element binding protein
mPFC	Medial prefrontal cortex
NAc	Nucleus accumbens
OVX	Ovariectomy
P	Postnatal day of age
SCH 1.0	1.0 µg/side dose of SCH 23390 (D1 receptor antagonist)
SKF 0.5	0.5 µg/side dose of SKF 81298 (D1 receptor agonist)
SKF 1.5	1.0 µg/side dose of SKF 81298 (D1 receptor agonist)
TH	Tyrosine hydoxylase
TH-ir	Tyrosine-hydroxylase immunoreactivity

Definitions of key terms

Agonist	A drug that mimics the effects of an endogenous ligand
Antagonist ligand	A drug that antagonizes or blocks the effects of an endogenous
CREB	cAMP response element binding protein. A constitutively expressed transcription factor that is involved in regulation of gene expression
c-fos	The gene that codes for the fos protein
fos	The protein product of the *c-fos* gene. Expression is upregulated in response to synaptic activity, thus making this gene a useful marker of neural activity
Intraperitoneal	Injection of a drug into the body cavity
Ligand	A molecule that binds to a receptor (e.g., dopamine)
Phosphorylation	A post-translational modification that alters activity of a protein
pCREB	Phosphorylated (the active form) of CREB
Raclopride	D2 dopamine receptor antagonist
Second messenger targets	Molecules that convey signals from membrane-bound receptors to inside the cell
SCH 23390	D1 dopamine receptor antagonist
SKF 81297	D1 dopamine receptor agonist
Tyrosine hydoxylase	The rate limiting enzyme in the synthesis of dopamine and other catecholamines. Used as an indirect index of dopamine levels

Brock University

Animal Care and Use Committee (ACUC)
Chair – Glenn Tattersall, PhD 905.688.5550 ext 4815
Clinical Veterinarian – Alistair Ker, D.V.M. 905.227.7644
Animal Care Technician – Dayle Belme, ACT, M.Sc. 905.688.5550 ext 3140

Date: Sep 20/07

Dear Dr. McCormick,

Your "Animal Use Project Proposal (AUPP)" entitled: **Is estrogen's enhancement of locomotor sensitisation to amphetamine mediated by corticosterone?**

has been approved by the Animal Care and Use Committee. This approval expires in one year on the last day of the month. The number for this project is **AUPP # 07 - 09 - 02.**
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED:
72 Long Evans rats

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Glenn Tattersall, Chair of ACUC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY.

Brock University

Animal Care and Use Committee (ACUC)
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Animal Care Technician – Dayle Belme, ACT, M.Sc. 905.688.5550 ext 3140

Date: Jan 15, 2008

Dear Dr. McCormick and Ms. Mathews,

Your "Animal Use Project Proposal (AUPP)" entitled: **The role of the developing mesocorticolimbic dopamine system in psychomotor stimulant reactivity in adolescence**

has been approved by the Animal Care and Use Committee. This approval expires in one year on the last day of the month. The number for this project is **AUPP # 07-11-01**
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: 240 Long Evans rats

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Glenn Tattersall, Chair of ACUC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY.

Brock University

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Animal Care Technician – Dayle Belme, ACT, M.Sc. 905.688.5550 ext 5820/3140

Date: Dec 18/08

Dear Dr. McCormick and Ms. Mathews

Your "Animal Use Project Proposal (AUPP)" entitled: **Adolescent development of sensitivity to acute and repeated amphetamine treatment.**

has been approved by the Animal Care and Use Committee. This approval expires in one year on the last day of the month. The number for this project is **AUPP # 08- 11- 01** .
This number must be indicated when ordering animals for this project.

ANIMALS APPROVED: **40 Long Evans rats**

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures, as described in this protocol, are familiar with the contents of this document.

Rene Vandenboom, Chair of ACUC

THIS PROTOCOL IS IN EFFECT FOR A PERIOD OF ONE YEAR ONLY.